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(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS

(57) Abstract

Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells *in situ* and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration *in vivo*. Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.

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DESCRIPTION

Methods and Compositions for Stimulating Bone Cells

5      The present application is a continuation-in-part of
U.S. Serial Number 08/316,650, filed September 30, 1994;
which is a continuation-in-part of U.S. Serial Number
08/199,780, filed February 18, 1994; the entire text and
figures of which disclosures are specifically
10   incorporated herein by reference without disclaimer.  The
United States government has certain rights in the
present invention pursuant to Grant HL-41926 from the
National Institutes of Health.

15   **1.  Field of the Invention**

     The present invention relates generally to the field
of bone cells and tissues.  More particularly, certain
embodiments concern the transfer of genetic material into
20   bone and other embodiments concern type II collagen.  In
certain examples, the invention concerns the use of type
II collagen and nucleic acids to stimulate bone growth,
repair and regeneration.  Methods, compositions, kits and
devices are provided for transferring an osteotropic gene
25   into bone progenitor cells, which is shown to stimulate
progenitor cells and to promote increased bone formation
*in vivo*.

     **2.  Description of the Related Art**
30

     Defects in the process of bone repair and
regeneration are linked to the development of several
human diseases and disorders, *e.g.*, osteoporosis and
osteogenesis imperfecta.  Failure of the bone repair
35   mechanism is, of course, also associated with significant
complications in clinical orthopaedic practice, for
example, fibrous non-union following bone fracture,

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implant interface failures and large allograft failures.
The lives of many individuals would be improved by the
development of new therapies designed to stimulate and
strengthen the fracture repair process.

5

   Naturally, any new technique to stimulate bone
repair would be a valuable tool in treating bone
fractures.  A significant portion of fractured bones are
still treated by casting, allowing natural mechanisms to
10  effect wound repair.  Although there have been advances
in fracture treatment in recent years, including improved
devices, the development of new processes to stimulate,
or complement, the wound repair mechanisms would
represent significant progress in this area.

15

   A very significant patient population that would
benefit from new therapies designed to promote fracture
repair, or even prevent or lessen fractures, are those
patients suffering from osteoporosis.  The term
20  osteoporosis refers to a heterogeneous group of disorders
characterized by decreased bone mass and fractures.
Clinically, osteoporosis is segregated into type I and
type II.  Type I osteoporosis occurs predominantly in
middle aged women and is associated with estrogen loss at
25  the menopause, while osteoporosis type II is associated
with advancing age.

   An estimated 20-25 million people are at increased
risk for fracture because of site-specific bone loss.
30  The cost of treating osteoporosis in the United States is
currently estimated to be in the order of $10 billion per
year.  Demographic trends, i.e., the gradually increasing
age of the US population, suggest that these costs may
increase 2-3 fold by the year 2020 if a safe and

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effective treatment is not found.

The major focus of current therapies for
osteoporosis is fracture prevention, not fracture repair.
5    This is an important consideration, as it is known that
significant morbidity and mortality are associated with
prolonged bed rest in the elderly, especially those who
have suffered hip fracture.  New methods are clearly
needed for stimulating fracture repair, thus restoring
10   mobility in these patients before the complications
arise.

Osteogenesis imperfecta (OI) refers to a group of
inherited connective tissue diseases characterized by
15   bone and soft connective tissue fragility (Byers and
Steiner, 1992; Prockop, 1990).  Males and females are
affected equally, and the overall incidence is currently
estimated to be 1 in 5,000-14,000 live births.  Hearing
loss, dentinogenesis imperfecta, respiratory
20   insufficiency, severe scoliosis and emphysema are just
some of the conditions that are associated with one or
more types of OI.  While accurate estimates of the health
care costs are not available, the morbidity and mortality
associated with OI certainly result from the extreme
25   propensity to fracture (OI types I-IV) and the
deformation of abnormal bone following fracture repair
(OI types II-IV) (Bonadio and Goldstein, 1993).  The most
relevant issue with OI treatment is to develop new
methods by which to improve fracture repair and thus to
30   improve the quality of life of these patients.

The techniques of bone reconstruction, such as is
used to reconstruct defects occurring as a result of
trauma, cancer surgery or errors in development, would
35   also be improved by new methods to promote bone repair.
Reconstructive methods currently employed, such as using
autologous bone grafts, or bone grafts with attached soft

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tissue and blood vessels, are associated with significant
drawbacks of both cost and difficulty.  For example,
harvesting a useful amount of autologous bone is not
easily achieved, and even autologous grafts often become
5    infected or suffer from resorption.

The process of bone repair and regeneration
resembles the process of wound healing in other tissues.
A typical sequence of events includes; hemorrhage; clot
10   formation; dissolution of the clot with concurrent
removal of damaged tissues; ingrowth of granulation
tissue; formation of cartilage; capillary ingrowth and
cartilage turnover; rapid bone formation (callus tissue);
and, finally, remodeling of the callus into cortical and
15   trabecular bone.  Therefore, bone repair is a complex
process that involves many cell types and regulatory
molecules.  The diverse cell populations involved in
fracture repair include stem cells, macrophages,
fibroblasts, vascular cells, osteoblasts, chondroblasts,
20   and osteoclasts.

Regulatory factors involved in bone repair are known
to include systemic hormones, cytokines, growth factors,
and other molecules that regulate growth and
25   differentiation.  Various osteoinductive agents have been
purified and shown to be polypeptide growth-factor-like
molecules.  These stimulatory factors are referred to as
bone morphogenetic or morphogenic proteins (BMPs), and
have also been termed osteogenic bone inductive proteins
30   or osteogenic proteins (OPs).  Several BMP (or OP) genes
have now been cloned, and the common designations are
BMP-1 through BMP-8.  New BMPs are in the process of
discovery.  Although the BMP terminology is widely used,
it may prove to be the case that there is an OP

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counterpart term for every individual BMP (Alper, 1994).

BMPs 2-8 are generally thought to be osteogenic,
although BMP-1 is a more generalized morphogen (Shimell
5    et al., 1991). BMP-3 is also called osteogenin (Luyten
et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et
al., 1990). BMPs are related to, or part of, the
transforming growth factor-$\beta$ (TGF-$\beta$) superfamily, and
both TGF-$\beta$1 and TGF-$\beta$2 also regulate osteoblast function
10   (Seitz et al., 1992). Several BMP (or OP) nucleotide
sequences and polypeptides have been described in U.S.
Patents, e.g., 4,795,804; 4,877,864; 4,968,590;
5,108,753; including, specifically, BMP-1 disclosed in
U.S. Patent 5,108,922; BMP-2A (currently referred to as
15   BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B
(currently referred to as BMP-4) disclosed in U.S. Patent
5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6
in 5,187,076; BMP-7 in 5,108,753 and 5,141,905; and OP-1,
COP-5 and COP-7 in 5,011,691.
20

Other growth factors or hormones that have been
reported to have the capacity to stimulate new bone
formation include acidic fibroblast growth factor
(Jingushi et al., 1990); estrogen (Boden et al., 1989);
25   macrophage colony stimulating factor (Horowitz et al.,
1989); and calcium regulatory agents such as parathyroid
hormone (PTH) (Raisz and Kream, 1983).

Several groups have investigated the possibility of
30   using bone stimulating proteins and polypeptides,
particularly recombinant BMPs, to influence bone repair
in vivo. For example, recombinant BMP-2 has been
employed to repair surgically created defects in the
mandible of adult dogs (Toriumi et al., 1991), and high
35   doses of this molecule have been shown to functionally
repair segmental defects in rat femurs (Yasko et al.,
1992). Chen and colleagues showed that a single

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application of 25-100 mg of recombinant TGF-β1 adjacent
to cartilage induced endochondral bone formation in the
rabbit ear full thickness skin wounds (Chen et al.,
1991). It has also been reported that an application of
5  TGF-β1 in a 3% methylcellulose gel was able to repair
surgically induced large skull defects that otherwise
heal by fibrous connective tissue and never form bone
(Beck et al., 1991).

10      However, there are many drawbacks associated with
these type of treatment protocols, not least the
expensive and time-consuming purification of the
recombinant proteins from their host cells. Also,
polypeptides, once administered to an animal are more
15  unstable than is generally desired for a therapeutic
agent, and they are susceptible to proteolytic attack.
Furthermore, the administration of recombinant proteins
can initiate various inhibitive or otherwise harmful
immune responses. It is clear, therefore, that a new
20  method capable of promoting bone repair and regeneration
in vivo would represent a significant scientific and
medical advance with immediate benefits to a large number
of patients. A method readily adaptable for use with a
variety of matrices and bone-stimulatory genes would be
25  particularly advantageous.

## SUMMARY OF THE INVENTION

30      The present invention overcomes one or more of these
and other drawbacks inherent in the prior art by
providing novel methods, compositions and devices for use
in transferring nucleic acids into bone cells and
tissues, and for promoting bone repair and regeneration.
35  Certain embodiments of the invention rest, generally,
with the inventors' surprising finding that nucleic acids
can be effectively transferred to bone progenitor cells

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*in vivo* and that, in certain embodiments, the transfer of an osteotropic gene stimulates bone repair in an animal.

The invention, in general terms, thus concerns
5    methods, compositions and devices for transferring a nucleic acid segment into bone progenitor cells or tissues. The methods of the invention generally comprise contacting bone progenitor cells with a composition comprising a nucleic acid segment in a manner effective
10   to transfer the nucleic acid segment into the cells. The cells may be cultured cells or recombinant cells maintained *in vitro*, when all that is required is to add the nucleic acid composition to the cells, *e.g.*, by adding it to the culture media.
15

Alternatively, the progenitor cells may be located within a bone progenitor tissue site of an animal, when the nucleic acid composition would be applied to the site in order to effect, or promote, nucleic acid transfer
20   into bone progenitor cells *in vivo*. In transferring nucleic acids into bone cells within an animal, a preferred method involves first adding the genetic material to a bone-compatible matrix and then using the resultant matrix to contact an appropriate tissue site
25   within the animal. The "resultant" matrix may, in certain embodiments, be referred to as a matrix impregnated with genetic material, or it may take the form of a matrix-nucleic acid mixture, or even conjugate.

30   An extremely wide variety of genetic material can be transferred to bone progenitor cells or tissues using the compositions and methods of the invention. For example, the nucleic acid segment may be DNA (double or single-stranded) or RNA (*e.g.*, mRNA, tRNA, rRNA); it may also be
35   a "coding segment", *i.e.*, one that encodes a protein or polypeptide, or it may be an antisense nucleic acid molecule, such as antisense RNA that may function to

disrupt gene expression. The nucleic acid segments may thus be genomic sequences, including exons or introns alone or exons and introns, or coding cDNA regions, or in fact any construct that one desires to transfer to a bone

5    progenitor cell or tissue. Suitable nucleic acid segments may also be in virtually any form, such as naked DNA or RNA, including linear nucleic acid molecules and plasmids; functional inserts within the genomes of various recombinant viruses, including viruses with DNA

10   genomes and retroviruses; and any form of nucleic acid segment, plasmid or virus associated with a liposome or a gold particle, the latter of which may be employed in connection with the gene gun technology.

15        The invention may be employed to promote expression of a desired gene in bone cells or tissues and to impart a particular desired phenotype to the cells. This expression could be increased expression of a gene that is normally expressed (i.e., "over-expression"), or it

20   could be used to express a gene that is not normally associated with bone progenitor cells in their natural environment. Alternatively, the invention may be used to suppress the expression of a gene that is naturally expressed in such cells and tissues, and again, to change

25   or alter the phenotype. Gene suppression may be a way of expressing a gene that encodes a protein that exerts a down-regulatory function, or it may utilize antisense technology.

30   **1.   Bone Progenitor Cells and Tissues**

         In certain embodiments, this invention provides advantageous methods for using genes to stimulate bone progenitor cells. As used herein, the term "bone

35   progenitor cells" refers to any or all of those cells that have the capacity to ultimately form, or contribute to the formation of, new bone tissue. This includes

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various cells in different stages of differentiation,
such as, for example, stem cells, macrophages,
fibroblasts, vascular cells, osteoblasts, chondroblasts,
osteoclasts, and the like.  Bone progenitor cells also
5    include cells that have·been isolated and manipulated *in
vitro*, *e.g.*, subjected to stimulation with agents such as
cytokines or growth factors or even genetically
engineered cells.  The particular type or types of bone
progenitor cells that are stimulated using the methods
10   and compositions of the invention are not important, so
long as the cells are stimulated in such a way that they
are activated and, in the context of *in vivo* embodiments,
ultimately give rise to new bone tissue.

15       The term "bone progenitor cell" is also used to
particularly refer to those cells that are located
within, are in contact with, or migrate towards (*i.e.*,
"home to"), bone progenitor tissue and which cells
directly or indirectly stimulate the formation of mature
20   bone.  As such, the progenitor cells may be cells that
ultimately differentiate into mature bone cells
themselves, *i.e.*, cells that "directly" form new bone
tissue.  Cells that, upon stimulation, attract further
progenitor cells or promote nearby cells to differentiate
25   into bone-forming cells (*e.g.*, into osteoblasts,
osteocytes and/or osteoclasts) are also considered to be
progenitor cells in the context of this disclosure - as
their stimulation "indirectly" leads to bone repair or
regeneration.  Cells affecting bone formation indirectly
30   may do so by the elaboration of various growth factors or
cytokines, or by their physical interaction with other
cell types.  Although of scientific interest, the direct
or indirect mechanisms by which progenitor cells

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stimulate bone or wound repair is not a consideration in
practicing this invention.

Bone progenitor cells and bone progenitor tissues
5    may be cells and tissues that, in their natural
environment, arrive at an area of active bone growth,
repair or regeneration (also referred to as a wound
repair site). In terms of bone progenitor cells, these
may also be cells that are attracted or recruited to such
10   an area. These may be cells that are present within an
artificially-created osteotomy site in an animal model,
such as those disclosed herein. Bone progenitor cells
may also be isolated from animal or human tissues and
maintained in an *in vitro* environment. Suitable areas of
15   the body from which to obtain bone progenitor cells are
areas such as the bone tissue and fluid surrounding a
fracture or other skeletal defect (whether or not this is
an artificially created site), or indeed, from the bone
marrow. Isolated cells may be stimulated using the
20   methods and compositions disclosed herein and, if
desired, be returned to an appropriate site in an animal
where bone repair is to be stimulated. In such cases,
the nucleic-acid containing cells would themselves be a
form of therapeutic agent. Such *ex vivo* protocols are
25   well known to those of skill in the art.

In important embodiments of the invention, the bone
progenitor cells and tissues will be those cells and
tissues that arrive at the area of bone fracture or
30   damage that one desires to treat. Accordingly, in
treatment embodiments, there is no difficulty associated
with the identification of suitable target progenitor
cells to which the present therapeutic compositions
should be applied. All that is required in such cases is
35   to obtain an appropriate stimulatory composition, as
disclosed herein, and contact the site of the bone
fracture or defect with the composition. The nature of

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this biological environment is such that the appropriate
cells will become activated in the absence of any further
targeting or cellular identification by the practitioner.

5       Certain methods of the invention involve, generally,
contacting bone progenitor cells with a composition
comprising one or more osteotropic genes (with or without
additional genes, proteins or other biomolecules) so as
to promote expression of said gene in said cells.  As
10      outlined above, the cells may be contacted *in vitro* or *in
vivo*.  This is achieved, in the most direct manner, by
simply obtaining a functional osteotropic gene construct
and applying the construct to the cells.  The present
inventors surprisingly found that there are no particular
15      molecular biological modifications that need to be
performed in order to promote effective expression of the
gene in progenitor cells.  Contacting the cells with DNA,
*e.g.*, a linear DNA molecule, or DNA in the form of a
plasmid or other recombinant vector, that contains the
20      gene of interest under the control of a promoter, along
with the appropriate termination signals, is sufficient
to result in uptake and expression of the DNA, with no
further steps necessary.

25      In preferred embodiments, the process of contacting
the progenitor cells with the osteotropic gene
composition is conducted *in vivo*.  Again, a direct
consequence of this process is that the cells take up and
express the gene and that they, without additional steps,
30      function to stimulate bone tissue growth, repair or
regeneration.

        An assay of an osteoinductive gene may be conducted
using the bone induction bioassay of Sampath and Reddi
35      (1981; incorporated herein by reference).  This is a rat
bone formation assay that is routinely used to evaluate
the osteogenic activity of bone inductive factors.

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However, for analyzing the effects of osteotropic genes
on bone growth, one is generally directed to use the
novel osteotomy model disclosed herein.

5    2.    Osteotropic Genes

        As used herein, the terms "osteotropic and
osteogenic gene" are used to refer to a gene or DNA
coding region that encodes a protein, polypeptide or
10    peptide that is capable of promoting, or assisting in the
promotion of, bone formation, or one that increases the
rate of primary bone growth or healing (or even a gene
that increases the rate of skeletal connective tissue
growth or healing). The terms promoting, inducing and
15    stimulating are used interchangeably throughout this text
to refer to direct or indirect processes that ultimately
result in the formation of new bone tissue or in an
increased rate of bone repair. Thus, an osteotropic gene
is a gene that, when expressed, causes the phenotype of a
20    cell to change so that the cell either differentiates,
stimulates other cells to differentiate, attracts bone-
forming cells, or otherwise functions in a manner that
ultimately gives rise to new bone tissue.

25        In using the new osteotomy model of the invention,
an osteotropic gene is characterized as a gene that is
capable of stimulating proper bone growth in the
osteotomy gap to any degree higher than that observed in
control studies, e.g., parallel studies employing an
30    irrelevant marker gene such as $\beta$-galactosidase. This
stimulation of "proper bone growth" includes both the
type of tissue growth and the rate of bone formation. In
using the model with a 5 mm osteotomy gap, an osteotropic
gene is generally characterized as a gene that is capable
35    of promoting or inducing new bone formation, rather than
abnormal bone fracture repair, i.e., fibrous non-union.
In using the 2 mm osteotomy gap, one may characterize

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osteotropic genes as genes that increase the rate of
primary bone healing as compared to controls, and more
preferably, genes capable of stimulating repair of the
osteotomy defect in a time period of less than nine
5    weeks.

In general terms, an osteotropic gene may also be
characterized as a gene capable of stimulating the growth
or regeneration of skeletal connective tissues such as,
10   e.g., tendon, cartilage, and ligament. Thus, in certain
embodiments, the methods and compositions of the
invention may be employed to stimulate the growth or
repair of both bone tissue itself and also of skeletal
connective tissues.
15

A variety of osteotropic genes are now known, all of
which are suitable for use in connection with the present
invention. Osteotropic genes and the proteins that they
encode include, for example, systemic hormones, such as
20   parathyroid hormone (PTH) and estrogen; many different
growth factors and cytokines; chemotactic or adhesive
peptides or polypeptides; molecules such as activin (U.S.
Patent 5,208,219, incorporated herein by reference);
specific bone morphogenetic proteins (BMPs); and even
25   growth factor receptor genes.

Examples of suitable osteotropic growth factors
include those of the transforming growth factor (TGF)
gene family, including TGFs 1-3, and particularly TGF-$\beta$1,
30   TGF-$\beta$2 and TGF-$\beta$3, (U.S. Patents 4,886,747 and 4,742,003,
incorporated herein by reference), with TGF-$\alpha$ (U.S.
Patent 5,168,051, incorporated herein by reference) also
being of possible use; and also fibroblast growth factors
(FGF), previously referred to as acidic and basic FGF and
35   now referred to as FGF1-9; granulocyte/macrophage colony
stimulating factor (GMCSF); epidermal growth factor
(EGF); platelet derived growth factor (PDGF); insulin-

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like growth factors (IGF), including IGF-I and IGF-II;
and leukemia inhibitory factor (LIF), also known as HILDA
and DIA.  Any. of the above or other related genes, or DNA
segments encoding the active portions of such proteins,
5     may be used in the novel methods and compositions of the
invention.

Certain preferred osteotropic genes and DNA segments
are those of the TGF superfamily, such as TGF-$\beta$1, TGF-$\beta$2,
10    TGF-$\beta$3 and members of the BMP family of genes. For
example, several BMP genes have been cloned that are
ideal candidates for use in the nucleic acid transfer or
delivery protocols of the invention.  Suitable BMP genes
are those designated BMP-2 through BMP-12.  BMP-1 is not
15    considered to be particularly useful at this stage.

There is considerable variation in the terminology
currently employed in the literature in referring to
these genes and polypeptides.  It will be understood by
20    those of skill in the art that all BMP genes that encode
an active osteogenic protein are considered for use in
this invention, regardless of the differing terminology
that may be employed.  For example, BMP-3 is also called
osteogenin and BMP-7 is also called OP-1 (osteogenic
25    protein-1).  It is likely that the family of factors
termed OP(s) is as large as that termed BMP(s), and that
these terms, in fact, describe the same set of molecules
(Alper, 1994).

30    The DNA sequences for several BMP (or OP) genes have
been described both in scientific articles and in U.S.
Patents such as 4,877,864; 4,968,590; 5,108,753.
Specifically, BMP-1 sequences are disclosed in U.S.
Patent 5,108,922; BMP-2A (currently referred to as BMP-2)
35    in U.S. Patents 5,166,058 and 5,013,649; BMP-2B
(currently referred to as BMP-4) disclosed in U.S. Patent
5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6

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in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all
incorporated herein by reference). The article by Wozney
et al., (1988; incorporated herein by reference) is
considered to be particularly useful for describing BMP
5    molecular clones and their activities. DNA sequences
encoding the osteogenic proteins designated OP-1, COP-5
and COP-7 are also disclosed in U.S. Patent 5,011,691.

All of the above issued U.S. Patents are
10    incorporated herein by reference and are intended to be
used in order to supplement the present teachings
regarding the preparation of BMP and OP genes and DNA
segments that express osteotropic polypeptides. As
disclosed in the above patents, and known to those of
15    skill in the art, the original source of a recombinant
gene or DNA segment to be used in a therapeutic regimen
need not be of the same species as the animal to be
treated. In this regard, it is contemplated that any
recombinant PTH, TGF or BMP gene may be employed to
20    promote bone repair or regeneration in a human subject or
an animal, e.g., a horse. Particularly preferred genes
are those from human, murine and bovine sources, in that
such genes and DNA segments are readily available, with
the human or murine forms of the gene being most
25    preferred for use in human treatment regimens.
Recombinant proteins and polypeptides encoded by isolated
DNA segments and genes are often referred to with the
prefix "r" for recombinant and "rh" for recombinant
human. As such, DNA segments encoding rBMPs, such as
30    rhBMP-2 or rhBMP-4, are contemplated to be particularly
useful in connection with this invention.

The definition of a "BMP gene", as used herein, is a
gene that hybridizes, under relatively stringent
35    hybridization conditions (see, e.g., Maniatis et al.,
1982), to DNA sequences presently known to include BMP
gene sequences.

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        To prepare an osteotropic gene segment or cDNA one
may follow the teachings disclosed herein a ` also the
teachings of any of patents or scientific documents
specifically referenced herein. Various nucleotide
5    sequences encoding active BMPs are disclosed in U.S.
Patents 5,166,058, 5,013,649, 5,116,738, 5,106,748,
5,187,076, 5,108,753 and 5,011,691, each incorporated
herein by reference. By way of example only, U.S. Patent
5,166,058, teaches that hBMP-2 is encoded by a nucleotide
10   sequence from nucleotide #356 to nucleotide #1543 of the
sequence shown in Table II of the patent. One may thus
obtain a hBMP-2 DNA segment using molecular biological
techniques, such as polymerase chain reaction (PCR™) or
screening a cDNA or genomic library, using primers or
15   probes with sequences based on the above nucleotide
sequence. The practice of such techniques is a routine
matter for those of skill in the art, as taught in
various scientific articles, such as Sambrook et al.,
(1989), incorporated herein by reference. Certain
20   documents further particularly describe suitable
mammalian expression vectors, e.g., U.S. Patent
5,168,050, incorporated herein by reference.

        Osteotropic genes and DNA segments that are
25   particularly preferred for use in certain aspects of the
present compositions and methods are the TGF, PTH and BMP
genes. TGF genes are described in U.S. Patents
5,168,051; 4,886,747 and 4,742,003, each incorporated
herein by reference. TGFα may not be as widely
30   applicable as TGFβ, but is proposed for use particularly
in applications involving skeletal soft tissues. The PTH
gene, or a DNA segment encoding the active fragment
thereof, such as a DNA segment encoding a polypeptide
that includes the amino acids 1-34 (hPTH1-34; Hendy et
35   al., 1981; incorporated herein by reference) is another

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preferred gene; as are the BMP genes termed BMP-4 and
BMP-2, such as the gene or cDNA encoding the murine BMP-4
disclosed herein.

5       It is also contemplated that one may clone further
genes or cDNAs that encode an osteotropic protein or
polypeptide.  The techniques for cloning DNA molecules,
i.e., obtaining a specific coding sequence from a DNA
library that is distinct from other portions of DNA, are
10      well known in the art.  This can be achieved by, .for
example, screening an appropriate DNA library, as
disclosed in Example XV herein, which relates to the
cloning of a wound healing gene.  The screening procedure
may be based on the hybridization of oligonucleotide
15      probes, designed from a consideration of portions of the
amino acid sequence of known DNA sequences encoding
related osteogenic proteins.  The operation of such
screening protocols are well known to those of skill in
the art and are described in detail in the scientific
20      literature, for example, in Sambrook et al., (1989),
incorporated herein by reference.

        Osteotropic genes with sequences that vary from
those described in the literature are also encompassed by
25      the invention, so long as the altered or modified gene
still encodes a protein that functions to stimulate bone
progenitor cells in any direct or indirect manner.  These
sequences include those caused by point mutations, those
due to the degeneracies of the genetic code or naturally
30      occurring allelic variants, and further modifications
that have been introduced by genetic engineering, i.e.,
by the hand of man.

        Techniques for introducing changes in nucleotide
35      sequences that are designed to alter the functional
properties of the encoded proteins or polypeptides are
well known in the art, e.g., U.S. Patent 4,518,584,

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incorporated herein by reference, which techniques are
also described in further detail herein.  Such
modifications include the deletion, insertion or
substitution of bases, and thus, changes in the amino
5     acid sequence.  Changes may be made to increase the
osteogenic activity of a protein, to increase its
biological stability or half-life, to change its
glycosylation pattern, and the like.  All such
modifications to the nucleotide sequences are encompassed
10    by this invention.

     It will, of course, be understood that one or more
than one osteotropic gene may be used in the methods and
compositions of the invention.  The nucleic acid delivery
15    methods may thus entail the administration of one, two,
three, or more, osteotropic genes.  The maximum number of
genes that may be applied is limited only by practical
considerations, such as the effort involved in
simultaneously preparing a large number of gene
20    constructs or even the possibility of eliciting a
significant adverse cytotoxic effect.  The particular
combination of genes may be two or more distinct BMP
genes; or it may be such that a growth factor gene is
combined with a hormone gene, e.g., a BMP gene and a PTH
25    gene; a hormone or growth factor gene may even be
combined with a gene encoding a cell surface receptor
capable of interacting with the polypeptide product of
the first gene.

30    In using multiple genes, they may be combined on a
single genetic construct under control of one or more
promoters, or they may be prepared as separate constructs
of the same of different types.  Thus, an almost endless
combination of different genes and genetic constructs may
35    be employed.  Certain gene combinations may be designed
to, or their use may otherwise result in, achieving
synergistic effects on cell stimulation and bone growth,

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any and all such combinations are intended to fall within
the scope of the present invention.  Indeed, many
synergistic effects have been described in the scientific
literature, so that one of ordinary skill in the art
5    would readily be able to·identify likely synergistic gene
combinations, or even gene-protein combinations.

It will also be understood that, if desired, the
nucleic segment or gene could be administered in
10   combination with further agents, such as, e.g., proteins
or polypeptides or various pharmaceutically active
agents.  So long as genetic material forms part of the
composition, there is virtually no limit to other
components which may also be included, given that the
15   additional agents do not cause a significant adverse
effect upon contact with the target cells or tissues.
The nucleic acids may thus be delivered along with
various other agents, for example, in certain embodiments
one may wish to administer an angiogenic factor, and/or
20   an inhibitor of bone resorption, as disclosed in U.S.
Patents 5,270,300 and 5,118,667, respectively, each
incorporated herein by reference.

3.    Gene Constructs and DNA Segments
25   .

As used herein, the terms "gene" and "DNA segment"
are both used to refer to a DNA molecule that has been
isolated free of total genomic DNA of a particular
species.  Therefore, a gene or DNA segment encoding an
30   osteotropic gene refers to a DNA segment that contains
sequences encoding an osteotropic protein, but is
isolated away from, or purified free from, total genomic
DNA of the species from which the DNA is obtained.
Included within the term "DNA segment", are DNA segments
35   and smaller fragments of such segments, and also
recombinant vectors, including, for example, plasmids,
cosmids, phage, retroviruses, adenoviruses, and the like.

- 20 -

The term "gene" is used for simplicity to refer to a
functional protein or peptide encoding unit. As will be
understood by those in the art, this functional term
includes both genomic sequences and cDNA sequences.

5    "Isolated substantially away from other coding sequences"
means that the gene of interest, in this case, an
osteotropic gene, forms the significant part of the
coding region of the DNA segment, and that the DNA
segment does not contain large portions of naturally-

10   occurring coding DNA, such as large chromosomal fragments
or other functional genes or cDNA coding regions. Of
course, this refers to the DNA segment as originally
isolated, and does not exclude genes or coding regions,
such as sequences encoding leader peptides or targeting

15   sequences, later added to the segment by the hand of man.

This invention provides novel ways in which to
utilize various known osteotropic DNA segments and
recombinant vectors. As described above, many such

20   vectors are readily available, one particular detailed
example of a suitable vector for expression in mammalian
cells is that described in U.S. Patent 5,168,050,
incorporated herein by reference. However, there is no
requirement that a highly purified vector be used, so

25   long as the coding segment employed encodes a osteotropic
protein and does not include any coding or regulatory
sequences that would have a significant adverse effect on
bone progenitor cells. Therefore, it will also be
understood that useful nucleic acid sequences may include

30   additional residues, such as additional non-coding
sequences flanking either of the 5' or 3' portions of the
coding region or may include various internal sequences,
i.e., introns, which are known to occur within genes.

35       After identifying an appropriate osteotropic gene or
DNA molecule, it may be inserted into any one of the many
vectors currently known in the art, so that it will

direct the expression and production of the osteotropic
protein when incorporated into a bone progenitor cell.
In a recombinant expression vector, the coding portion of
the DNA segment is positioned under the control of a

5      promoter.  The promoter may be in the form of the
promoter which is naturally associated with an
osteotropic gene, as may be obtained by isolating the 5'
non-coding sequences located upstream of the coding
segment or exon, for example, using recombinant cloning

10     and/or PCR™ technology, in connection with the
compositions disclosed herein.


In other embodiments, it is contemplated that
certain advantages will be gained by positioning the

15     coding DNA segment under the control of a recombinant, or
heterologous, promoter.  As used herein, a recombinant or
heterologous promoter is intended to refer to a promoter
that is not normally associated with an osteotropic gene
in its natural environment.  Such promoters may include

20     those normally associated with other osteotropic genes,
and/or promoters isolated from any other bacterial,
viral, eukaryotic, or mammalian cell.  Naturally, it will
be important to employ a promoter that effectively
directs the expression of the DNA segment in bone

25     progenitor cells.


The use of recombinant promoters to achieve protein
expression is generally known to those of skill in the
art of molecular biology, for example, see Sambrook et

30     al., (1989).  The promoters employed may be constitutive,
or inducible, and can be used under the appropriate
conditions to direct high level or regulated expression
of the introduced DNA segment.  The currently preferred
promoters are those such as CMV, RSV LTR, the SV40

35     promoter alone, and the SV40 promoter in combination with
various enhancer elements.

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Osteotropic genes and DNA segments may also be in the form of a DNA insert which is located within the genome of a recombinant virus, such as, for example a recombinant adenovirus, adeno-associated virus (AAV) or
5    retrovirus.  In such embodiments, to place the gene in contact with a bone progenitor cell, one would prepare the recombinant viral particles, the genome of which includes the osteotropic gene insert, and simply contact the progenitor cells or tissues with the virus, whereby
10   the virus infects the cells and transfers the genetic material.

In certain preferred embodiments, one would impregnate a matrix or implant material with virus by
15   soaking the material in recombinant virus stock solution, e.g., for 1-2 hours, and then contact the bone progenitor cells or tissues with the resultant, impregnated matrix. Cells then penetrate, or grow into, the matrix, thereby contacting the virus and allowing viral infection which
20   leads to the cells taking up the desired gene or cDNA and expressing the encoded protein.

In other preferred embodiments, one would form a matrix-nucleic acid admixture, whether using naked DNA, a
25   plasmid or a viral vector, and contact the bone progenitor cells or tissues with the resultant admixed matrix.  The matrix may then deliver the nucleic acid into the cells following disassociation at the cell surface, or in the immediate cellular environment.
30   Equally, the matrix admixture itself, especially a particle- or fiber-DNA admixture, may be taken up by cells to provide subsequent intracellular release of the genetic material.  The matrix may then be extruded from the cell, catabolized by the cell, or even stored within
35   the cell.  The molecular mechanism by which a bone-compatible matrix achieves transfer of DNA to a cell is immaterial to the practice of the present invention.

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4.    Bone-Compatible Matrices

In certain preferred embodiments, the methods of the
invention involved preparing a composition in which the
osteotropic gene, genes, DNA segments, or cells already
incorporating such genes or segments, are associated
with, impregnated within, or even conjugated to, a bone-
compatible matrix, to form a "matrix-gene composition"
and the matrix-gene composition is then placed in contact
with the bone progenitor cells or tissue.  The matrix may
become impregnated with a gene DNA segment simply by
soaking the matrix in a solution containing the DNA, such
as a plasmid solution, for a brief period of time of
anywhere from about 5 minutes or so, up to and including
about two weeks.

Matrix-gene compositions are all those in which
genetic material is adsorbed, absorbed, impregnated,
conjugated to, or otherwise generally maintained in
contact with the matrix.  "Maintained in contact with the
matrix" means that an effective amount of the nucleic
acid composition should remain functionally associated
with the matrix until its transfer to the bone progenitor
cell or its release in the bone tissue site.

The type of matrix that may be used in the
compositions, devices and methods of the invention is
virtually limitless, so long as it is a "bone-compatible
matrix".  This means that the matrix has all the features
commonly associated with being "biocompatible", in that
it is in a form that does not produce a significant
adverse, allergic or other untoward reaction when
administered to an animal, and that it is also suitable
for placing in contact with bone tissue.  A "significant"
adverse effect is one that exceeds the normally accepted
side-effects associated with any given therapy.

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"Bone-compatible", as used herein, means that the
matrix (and gene) does not produce a significant adverse
or untoward reaction when placed in contact with bone.
In certain embodiments, when electing to use a particular
5    bone compatible matrix, one may, optionally, take various
other factors into consideration, for example, the
capacity of the matrix to provide a structure for the
developing bone, its capacity to be resorbed into the
body after the bone has been repaired, and such like.
10   However, these properties are not required to practice
the invention and are merely exemplary of the factors
that may be considered.

In other embodiments, one may also consider the
15   likelihood that the matrix will be transported into the
cell, e.g., by active or passive membrane transport.
Where such transport and subsequent nucleic acid release
is contemplated, other properties of the matrix and gene
may be assessed in optimizing the matrix-gene
20   formulation.  For example, adenovirus vectors may provide
for advantageous DNA release in such embodiments.
Matrices that are readily metabolized in the cytoplasm
would also likely be preferred in such embodiments.
Matrices that are later released from the cell, and
25   preferably, also removed from the surrounding tissue
area, would be another preferred form of matrix for use
in such embodiments.

The choice of matrix material will differ according
30   to the particular circumstances and the site of the bone
that is to be treated.  Matrices such as those described
in U.S. Patent 5,270,300 (incorporated herein by
reference) may be employed.  Physical and chemical
characteristics, such as, e.g., biocompatibility,
35   biodegradability, strength, rigidity, interface
properties, and even cosmetic appearance, may be
considered in choosing a matrix, as is well known to

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those of skill in the art.  Appropriate matrices will
deliver the gene composition and, in certain
circumstances, may be incorporated into a cell, or may
provide a surface for new bone growth, *i.e.*, they may act
5    as an *in situ* scaffolding through which progenitor cells
may migrate.

A particularly important aspect of the present
invention is its use in connection with orthopaedic
10   implants and interfaces and artificial joints, including
implants themselves and functional parts of an implant,
such as, *e.g.*, surgical screws, pins, and the like.  In
preferred embodiments, it is contemplated that the metal
surface or surfaces of an implant or a portion thereof,
15   such as a titanium surface, will be coated with a
material that has an affinity for nucleic acids, most
preferably, with hydroxyl apatite, and then the coated-
metal will be further coated with the gene or nucleic
acid that one wishes to transfer.  The available chemical
20   groups of the absorptive material, such as hydroxyl
apatite, may be readily manipulated to control its
affinity for nucleic acids, as is known to those of skill
in the art.

25   In certain embodiments, non-biodegradable matrices
may be employed, such as sintered hydroxylapatite,
aluminates, other bioceramic materials and metal
materials, particularly titanium.  A suitable ceramic
delivery system is that described in U.S. Patent
30   4,596,574, incorporated herein by reference.  Polymeric
matrices may also be employed, including acrylic ester
polymers, lactic acid polymers, and polylactic
polyglycolic acid (PLGA) block copolymers, have been
disclosed (U.S. Patent 4,526,909, U.S. Patent 4,563,489,
35   Simons *et al.*, 1992, and Langer and Folkman, 1976,
respectively, each incorporated herein by reference).

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In certain embodiments, it is contemplated that a
biodegradable matrix will likely be most useful.  A
biodegradable matrix is generally defined as one that is
capable of being resorbed into the body.  Potential

5    biodegradable matrices for use in connection with the
compositions, devices and methods of this invention
include, for example, biodegradable and chemically
defined calcium sulfate, tricalciumphosphate,
hydroxylapatite, PLGA block copolymers, polyanhydrides,

10   matrices of purified proteins, and semi-purified
extracellular matrix compositions.

One preferred group of matrices are collagenous
matrices, including those obtained from tendon or dermal

15   collagen, e.g., type I collagen, which is generally
prepared from dermis; those obtained from cartilage, such
as type II collagen; and various other types of collagen.
Collagens may be obtained from a variety of commercial
sources, e.g., Sigma that supplies type II collagen

20   obtained from bovine trachea; and Collagen Corporation.
Collagen matrices may also be prepared as described in
U.S. Patents 4,394,370 and 4,975,527, each incorporated
herein by reference.

25   The various collagenous materials may also be in the
form of mineralized collagen.  One preferred mineralized
collagenous material is that termed UltraFiber™,
obtainable from Norian Corp. (Mountain View, CA).  U.S.
Patent 5,231,169, incorporated herein by reference,

30   describes the preparation of mineralized collagen through
the formation of calcium phosphate mineral under mild
agitation in situ in the presence of dispersed collagen
fibrils.  Such a formulation may be employed in the

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context of delivering a nucleic acid segment to a bone
tissue site.

5        Certain other preferred collagenous materials are
those based upon type II·collagen.  Type II collagen
preparations have been discovered to have the surprising
and advantageous property of, absent any osteotropic
gene, being capable of stimulating bone progenitor cells.
Prior to the present invention, it was thought that
10       type II collagen only had a structural role in the
cartilage extracellular matrix and the present finding
that type II collagen is actually an
osteoconductive/osteoinductive material is unexpected.
The present invention thus contemplates the use of a
15       variety of type II collagen preparations as gene transfer
matrices or bone cell stimulants, either with or without
DNA segments, including native type II collagen, as
prepared from cartilage, and recombinant type II
collagen.
20

PLGA block copolymers may also be employed as gene
transfer matrices.  Such polymers have been shown to
readily incorporate DNA, are commercially available, non-
toxic, and hydrolyze at defined rates, (i.e. they
25       facilitate the sustained release of pharmaceutical
agents).  PLGA block copolymers have two particular
advantageous properties in that, first, they exhibit
reversible thermal gelation, and second, may be combined
with other agents that allow for radiographic
30       visualization.

5.   **Nucleic Acid Transfer Embodiments**

Once a suitable matrix-gene composition has been
35       prepared or obtained, all that is required to deliver the
osteotropic gene to bone progenitor cells within an
animal is to place the matrix-gene composition in contact

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with the site in the body in which one wishes to promote
bone growth.  This may be achieved by physically
positioning the matrix-gene composition in contact with
the body site, or by injecting a syringeable form of the
5    matrix-gene composition into the appropriate area.

The matrix-gene composition may be applied to a
simple bone fracture site that one wishes to repair, an
area of weak bone, such as in a patient with
10   osteoporosis, or a bone cavity site that one wishes to
fill with new bone tissue.  Bone cavities may arise as a
result of an inherited disorder, birth defect, or may
result following dental or periodontal surgery or after
the removal of an osteosarcoma.
15

The use of PLGA and like compounds as matrices
allows the matrix-DNA composition to be syringeable,
which is achieved by, generally, admixing the matrix-gene
composition with a pluronic agent.  The resultant matrix-
20   gene-pluronic may be stored within a thermal-jacket
syringe, maintained at a temperature of about 4°C,
immediately prior to administration to the body.  In this
temperature and environment, the composition will be a
liquid.  Following insertion into the body, the
25   composition will equilibrate towards body temperature,
and in so-doing will form a gelatinous matrix.

The above phenomenon is termed "reversible thermal
gelation", and this allows for a controlled rate of
30   gelation to be achieved.  The manner of using pluronic
agents in this context will be known to those of skill in
the art in light of the present disclosure.  Matrix-gene-
pluronic compositions may also be admixed, or generally
associated with, an imaging agent so that the present
35   gene transfer technology may be used in imaging
modalities.  In these cases, the attending physician or
veterinarian will be able to monitor the delivery and

positioning of the matrix-gene composition.  Many safe
and effective imaging agents, such as the radiographic
compound calcium phosphate, are available that may be
used in conjunction with fluoroscopy, or even with
5       tomography, to image the body or tissue site while the
composition is being delivered.

Where an image of the tissue site is to be provided,
one will desire to use a detectable imaging agent, such
10      as a radiographic agent, or even a paramagnetic or
radioactive agent.  Many radiographic diagnostic agents
are known in the art to be useful for imaging purposes,
including e.g., calcium phosphate.

15      In the case of paramagnetic ions, examples include
chromium (III), manganese (II), iron (III), iron (II),
cobalt (II), nickel (II), copper (II), neodymium (III),
samarium (III), ytterbium (III), gadolinium (III),
vanadium (II), terbium (III), dysprosium (III), holmium
20      (III) and erbium (III), with gadolinium being generally
preferred.  Ions useful in other contexts, such as X-ray
imaging, include but are not limited to, lanthanum (III),
gold (III), lead (II), and especially bismuth (III).

25      Although not generally preferred, radioactive
isotopes are not excluded and may be used for imaging
purposes if desired.  Suitable ions include iodine[131],
iodine[123], technicium[99m], indium[111], rhenium[188], rhenium[186],
gallium[67], copper[67], yttrium[90], iodine[125] and astatine[211].
30
The amount of gene construct that is applied to the
matrix and the amount of matrix-gene material that is
applied to the bone tissue will be determined by the
attending physician or veterinarian considering various
35      biological and medical factors.  For example, one would
wish to consider the particular osteotropic gene and
matrix, the amount of bone weight desired to be formed,

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the site of bone damage, the condition of the damaged
bone, the patient's or animal's age, sex, and diet, the
severity of any infection, the time of administration and
any further clinical factors that may affect bone growth,
5   such as serum levels of various factors and hormones.
The suitable dosage regimen will therefore be readily
determinable by one of skill in the art in light of the
present disclosure, bearing in mind the individual
circumstances.
10

In treating humans and animals, progress may be
monitored by periodic assessment of bone growth and/or
repair, e.g., using X-rays. The therapeutic methods and
compositions of the invention are contemplated for use in
15  both medical and veterinary applications, due to the lack
of species specificity in bone inductive factors. In
particular, it is contemplated that domestic, farm and
zoological animals, as well as thoroughbred horses, would
be treatable using the nucleic acid transfer protocols
20  disclosed herein.

The present methods and compositions may also have
prophylactic uses in closed and open fracture reduction
and also in the improved fixation of artificial joints.
25  The invention is applicable to stimulating bone repair in
congenital, trauma-induced, or oncologic resection-
induced craniofacial defects, and also is useful in the
treatment of periodontal disease and other tooth repair
processes and even in cosmetic plastic surgery. The
30  matrix-gene compositions and devices of this invention
may also be used in wound healing and related tissue
repair, including, but not limited to healing of burns,
incisions and ulcers.

35  The present invention also encompasses DNA-based
compositions for use in cellular transfer to treat bone
defects and disorders. The compositions of the invention

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generally comprise an osteotropic gene in association
with a bone-compatible matrix, such as type II collagen,
wherein the composition is capable of stimulating bone
growth, repair or regeneration upon administration to, or
5    implantation within, a bone progenitor tissue site of an
animal.   The osteotropic gene or genes may be any of
those described above, with TGF-$\alpha$ (for soft skeletal
tissues), TGF-$\beta$1, TGF-$\beta$2, TGF-$\beta$3, PTH, BMP-2 and BMP-4
genes being generally preferred.   Likewise, irrespective
10   of the choice of gene, the bone-compatible matrix may be
any of those described above, with biodegradable matrices
such as collagen and, more particularly, type II
collagen, being preferred.

15      In still further embodiments, the present invention
concerns osteotropic devices, which devices may be
generally considered as molded or designed matrix-gene
compositions.   The devices of the invention naturally
comprise a bone-compatible matrix in which an osteotropic
20   gene is associated with the matrix.   The combination of
genes and matrix components is such that the device is
capable of stimulating bone growth or healing when
implanted in an animal.   The devices may be of virtually
any size or shape, so that their dimensions are adapted
25   to fit a bone fracture or bone cavity site in the animal
that is to be treated, allowing the fracture join and/or
bone regrowth to be more uniform.   Other particularly
contemplated devices are those that are designed to act
as an artificial joint.   Titanium devices and
30   hydroxylapatite-coated titanium devices will be preferred
in certain embodiments.   Parts of devices in·combination
with an osteotropic nucleic acid segment, such as a DNA-
coated screw for an artificial joint, and the like, also
fall within the scope of the invention.
35

Therapeutic kits comprising, in suitable container
means, a bone compatible matrix, such as type II collagen

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or a PLGA block polymer, and an osteotropic gene form
another aspect of the invention. Such kits will
generally contain a pharmaceutically acceptable
formulation of the matrix and a pharmaceutically
5    acceptable formulation of an osteotropic gene, such as
PTH, BMP, TGF-$\beta$, FGF, GMCSF, EGF, PDGF, IGF or a LIF
gene. Currently preferred genes include PTH, TGF-$\beta$1,
TGF-$\beta$2, TGF-$\beta$3, and BMP-4 genes.

10        The kits may comprise a single container means that
contains both the biocompatible matrix and the
osteotropic gene. The container means may, if desired,
contain a pharmaceutically acceptable sterile syringeable
matrix, having associated with it, the osteotropic gene
15    composition and, optionally, a detectable label or
imaging agent. The syringeable matrix-DNA formulation
may be in the form of a gelatinous composition, e.g., a
type II collagen-DNA composition, or may even be in a
more fluid form that nonetheless forms a gel-like
20    composition upon administration to the body. In these
cases, the container means may itself be a syringe,
pipette, or other such like apparatus, from which the
matrix-DNA material may be applied to a bone tissue site
or wound area. However, the single container means may
25    contain a dry, or lyophilized, mixture of a matrix and
osteotropic gene composition, which may or may not
require pre-wetting before use.

        Alternatively, the kits of the invention may
30    comprise distinct container means for each component. In
such cases, one container would contain the osteotropic
gene, either as a sterile DNA solution or in a
lyophilized form, and the other container would include
the matrix, which may or may not itself be pre-wetted
35    with a sterile solution, or be in a gelatinous, liquid or
other syringeable form.

The kits may also comprise a second or third container means for containing a sterile, pharmaceutically acceptable buffer, diluent or solvent. Such a solution may be required to formulate either the

5   DNA component, the matrix component, both components separately, or a pre-mixed combination of the components, into a more suitable form for application to the body, e.g., a more gelatinous form. It should be noted, however, that all components of a kit could be supplied

10  in a dry form (lyophilized), which would allow for "wetting" upon contact with body fluids. Thus, the presence of any type of pharmaceutically acceptable buffer or solvent is not a requirement for the kits of the invention. The kits may also comprise a second or

15  third container means for containing a pharmaceutically acceptable detectable imaging agent or composition.

    The container means will generally be a container such as a vial, test tube, flask, bottle, syringe or

20  other container means, into which the components of the kit may placed. The matrix and gene components may also be aliquoted into smaller containers, should this be desired. The kits of the present invention may also include a means for containing the individual containers

25  in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials or syringes are retained.

    Irrespective of the number of containers, the kits

30  of the invention may also comprise, or be packaged with, an instrument for assisting with the placement of the ultimate matrix-gene composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, or any such medically approved delivery vehicle.

35

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6.    Type II Collagen as an Osteoconductive/inductive
      Material

The present invention also provides methods for
stimulating bone progenitor cells, as may be applied, in
certain circumstances, to promote new bone formation, or
to stimulate wound-healing.  As such, the bone progenitor
cells that are the targets of the invention may also be
termed "wound healing bone progenitor cells".  Although
the function of wound healing itself may not always be
required to practice all aspects of the invention, and
although a mechanistic understanding is not necessary to
practice the invention, it is generally thought that the
wound healing process does operate during execution of
the invention.

To stimulate a bone progenitor cell in accordance
with these aspects of the invention, generally one would
contact a bone progenitor cell with a composition
comprising a biologically effective amount of type II
collagen.  Although preparations of crushed bone and
mineralized collagen have been shown to be
osteoconductive, this property has not previously been
ascribed to type II collagen.  The present inventors have
found that type II collagen alone is surprisingly
effective at promoting new bone formation, it being able
to bridge a 5 mm osteotomy gap in only eight weeks in all
animals tested (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG.
6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, and FIG.
8C).

The forms of type II collagen that may be employed
in this invention are virtually limitless.  For example,
type II collagen may be purified from hyaline cartilage
of bovine trachea, or as isolated from diarthrodial
joints or growth plates.  Purified type II collagen is
commercially available and may be purchased from, e.g.,
Sigma Chemical Company, St. Louis, MO.  Any form of

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recombinant type II collagen may also be employed, as may
be obtained from a type II collagen-expressing
recombinant host cell, including bacterial, yeast,
mammalian, and insect cells.  One particular example of a
5    recombinant type II collagen expression system is a yeast
cell that includes an expression vector that encodes type
II collagen, as disclosed herein in Example VI.

     The type II collagen used in the invention may, if
10   desired, be supplemented with additional minerals, such
as calcium, e.g., in the form of calcium phosphate.  Both
native and recombinant type II collagen may be
supplemented by admixing, adsorbing, or otherwise
associating with, additional minerals in this manner.
15   Such type II collagen preparations are clearly
distinguishable from the types of "mineralized collagen"
previously described, e.g., in U.S. Patent 5,231,169 that
describes the preparation of mineralized total collagen
fibrils.
20

     An object of this aspect of the invention is to
provide a source of osteoconductive matrix material, that
may be reproducibly prepared in a straightforward and
cost-effective manner, and that may be employed, with or
25   without an osteotropic gene segment, to stimulate bone
progenitor cells.  Recombinant type II collagen was
surprisingly found to satisfy these criteria.  Although
clearly not required for effective results, the
combination of native or recombinant type II collagen
30   with mineral supplements, such as calcium, is encompassed
by this invention.

     A biologically effective amount of type II collagen
is an amount of type II collagen that functions to
35   stimulate a bone progenitor cell, as described herein.
By way of example, one measure of a biologically
effective amount is an amount effective to stimulate bone

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progenitor cells to the extent that new bone formation is evident. In this regard, the inventors have shown that 10 mg of lyophilized collagen functions effectively to close a 5 mm osteotomy gap in three weeks. This information may be used by those of skill in the art to optimize the amount of type II collagen needed for any given situation.

Depending on the individual case, the artisan would, in light of this disclosure, readily be able to calculate an appropriate amount, or dose, of type II collagen for stimulating bone cells and promoting bone growth. In terms of small animals or human subjects, suitable effective amounts of collagen include between about 1 mg and about 500 mg, and preferably, between about 1 mg and about 100 mg, of lyophilized type II collagen per bone tissue site. Of course, it is likely that there will be variations due to, e.g., individual responses, particular tissue conditions, and the speed with which bone formation is required. While 10 mg were demonstrated to be useful in the illustrative example, the inventors contemplate that 1, 5, 10, 15, 20, 30, 40, 50, 75, 100, 125, 150, 200, 300 mg, and the like, may be usefully employed for human patients and small animals. Of course, any values within these contemplated ranges may be useful in any particular case.

Naturally, one of the main variables to be accounted for is the amount of new bone that needs to be generated in a particular area or bone cavity. This can be largely a function of the size of the animal to be treated, e.g., a cat or a horse. Therefore, there is currently no upper limit on the amount of type II collagen, or indeed on the amount of any matrix-gene composition, that can be employed in the methods of the invention, given careful supervision by the practitioner.

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In contacting or applying type II collagen, with or without a DNA segment, to bone progenitor cells located within a bone progenitor tissue site of an animal, bone tissue growth will be stimulated. Thus, bone cavity
5   sites and bone fractures may be filled and repaired.

The use of type II collagen in combination with a nucleic acid segment that encodes a polypeptide or protein that stimulates bone progenitor cells when
10   expressed in said cells is preferred, as described above. Nucleic acid segments that comprise an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or a chemotactic factor gene are preferred, with PTH, TGF-$\beta$ and BMP genes being most
15   preferred. The genes function subsequent to their transfer into, and expression in, bone progenitor cells of the treated animal, thus promoting bone growth.

Although type II collagen alone is effective, its
20   combined use with an osteotropic gene segment may prove to give synergistic and particularly advantageous effects. Type II collagen, whether native or recombinant, may thus also be formulated into a therapeutic kit with an osteotropic gene segment, in
25   accordance with those kits described herein above. This includes the use of single or multiple container means, and combination with any medically approved delivery vehicle, including, but not limited to, syringes, pipettes, forceps, additional diluents, and the like.
30

## BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification
35   and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings

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in combination with the detailed description of specific
embodiments presented herein.

FIG. 1.  A model of DNA therapy for bone repair.

5

FIG. 2A.  A schematic model of the cellular and
molecular basis of the direct DNA transfer mechanism into
osteogenic cells *in vivo*.  Shown is the method of
creating osteotomy and placing gene-activated matrix *in*
10  *situ*.

FIG. 2B.  A schematic model of the cellular and
molecular basis of the direct DNA transfer mechanism into
osteogenic cells *in vivo*.  Shown is the method of
15  fracturing repair cells where blood vessels grow into the
gene-activated matrix (FIG. 2A).

FIG. 2C.  A schematic model of the cellular and
molecular basis of the direct DNA transfer mechanism into
20  osteogenic cells *in vivo*.  Shown are fractured cells
taking up DNA as an episomal element, *i.e.* direct gene
transfer *in vivo*.

FIG. 2D.  A schematic model of the cellular and
25  molecular basis of the direct DNA transfer mechanism into
osteogenic cells *in vivo*.  Shown are fractured repair
synthesizing and secreting recombinant proteins encoded
by the episomal DNA.

30  FIG. 2E.  A schematic model of the cellular and
molecular basis of the direct DNA transfer mechanism into
osteogenic cells *in vivo*.  Shown is the resulting new
bone formation.

35  FIG. 3A.  Achilles' tendon gene transfer is shown as
a time course overview at 3 weeks post-surgery.

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FIG. 3B. Achilles' tendon gene transfer is shown as a time course overview at 9 weeks post-surgery.

FIG. 3C. Achilles' tendon gene transfer is shown as
5   a time course overview at 12 weeks post-surgery.

FIG. 3D. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant
10   impregnated with expression plasmid DNA. Note the positive cytoplasmic staining of fibroblastic cells 9 weeks post-surgery.

FIG. 3E. Achilles' tendon gene transfer is shown as
15   a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant alone, without DNA. Note the relative absence of cytoplasmic staining.

20       FIG. 4. Monitoring of cruciate ligament gene transfer using a substrate utilization assay. Three weeks following the implantation of SIS soaked in a solution of the pSV40$\beta$-gal expression plasmid, tendon tissue was harvested, briefly fixed in 0.5%
25   glutaraldehyde, and then incubated with X-gal according to published methods. Tissues were then embedded in paraffin and sections were cut and stained with H and E. Note the positive (arrows) staining in the cytoplasm of granulation tissues fibroblasts.
30

       FIG. 5A. Direct DNA transfer into regenerating bone: $\beta$-gal activity. The figure compares $\beta$-galactosidase activity in homogenates of osteotomy gap tissue from two Sprague-Dawley rats. In animal #1, the
35   UltraFiber™ implant material was soaked in a solution of pSV40$\beta$-gal DNA, Promega) encoding bacterial $\beta$-galactosidase. In animal #2, the implant material was

- 40 -

soaked in a pure solution of pGL2-Promoter Vector DNA
(Promega) encoding insect luciferase. Enzyme activity
was determined using substrate assay kits ($\beta$-
galactosidase and Luciferase Assay Systems, Promega).
5    Note that significant $\beta$-galactosidase activity was found
only in the homogenate prepared from animal #1.


    FIG. 5B.  Direct DNA transfer into regenerating
bone: luciferase activity.  The figure compares
10   luciferase activity in aliquots of the homogenates
described in FIG. 5A.  Luciferase activity was determined
using the commercial reagents and protocols (Promega)
described in FIG. 5A.  Note that significant luciferase
activity is found only in the homogenate prepared from
15   animal #2.


    FIG. 6A.  Osteotomy gene transfer monitored by PTH
studies.  In this study an expression plasmid coding for
a functional 34 amino acid peptide fragment of human
20   parathyroid hormone (PTH1-34) was transferred and
expressed in vivo using the GAM technology.  The progress
of new bone formation in the gap was monitored
radiographically for three weeks and the animals were
sacrificed.  Shown is a radiograph of the osteotomy gap
25   of the control animal that received an antisense hPTH1-34
GAM construct.  There was no evidence of radiodense
tissue in the gap.


    FIG. 6B.  Osteotomy gene transfer (FIG. 6A)
30   monitored by PTH studies.  Shown is a histological
section of osteotomy repair tissue from the same control
animal.  The section is characterized by the presence of
granulation tissue fibroblasts and capillaries.


35   FIG. 6C.  Osteotomy gene transfer (FIG. 6A)
monitored by PTH studies.  Shown is a radiograph of the
osteotomy gap that received the sense PTH1-34 GAM

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construct.  Note the presence of radiodense tissue in the
gap (arrow).

5      FIG. 6D.  Osteotomy gene transfer (FIG. 6A)
monitored by PTH studies.  Shown is a histological
section of osteotomy repair tissue from the same control
animal.  The section is characterized by the presence of
trabecular bone plates that extend into the gap from the
surgical margin.

10

       FIG. 7A.  Osteotomy gene transfer BMP-4 studies.
Shown is immunohistochemical evidence of BMP-4 transgene
expression by granulation tissue fibroblasts near the
center of an osteotomy gap three weeks after surgery.

15     Note the positive (arrows) staining of spindled cells.
The BMP-4 transgene included an epitope tag (HA epitope,
Pharmacia) that facilitated the identification of
transgenic BMP-4 molecules.  Tissue staining was
performed using commercially available polyclonal anti-HA

20     antibodies and standard procedures.  Immunostaining was
localized only to gap tissues.  Control sections included
serial sections stained with pre-immune rabbit serum and
tissue sections from 13 control osteotomy gaps.  In both
instances all controls were negative for peroxidase

25     staining of granulation tissue fibroblasts.


       FIG. 7B.  Osteotomy gene transfer BMP-4 studies.
Shown is the histology of newly formed bone as early as
three weeks following gene transfer (FIG. 7A).

30

       FIG. 8A.  Radiographic evidence of bridging new bone
formation (arrows) as a consequence of BMP-4 gene
transfer and expression at six weeks' post surgery.  9
and 16 weeks post-op, are presented in FIG. 8B and FIG.

35     8C, respectively, to demonstrate the orderly growth of
new bone in situ over time.  This animal, which has been
maintained for 23 weeks, has been ambulating normally

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without an external fixator for the past 7 weeks.
Similar results have been obtained in a second long term
animal (of two) that is now 17 weeks post-op.

5          FIG. 8B.  Radiographic evidence of bridging new bone
formation (arrows) as a consequence of BMP-4 gene
transfer and expression at nine weeks' post surgery (see
FIG. 8A).

10         FIG. 8C.  Radiographic evidence of bridging new bone
formation (arrows) as a consequence of BMP-4 gene
transfer and expression at sixteen weeks' post surgery
(see FIG. 8A).

15         FIG. 9A.   The animal shown here is representative
of the control group that received an osteotomy plus a
collagen sponge without DNA of any type.  The animal was
maintained for 9 weeks following surgery and then
sacrificed.  Progress of new bone formation in the gap
20  was monitored radiographically and histologically.  Shown
is a radiograph of the osteotomy gap at 9 weeks.  Note
the absence of radiodense tissue in the gap.

           FIG. 9B.   Shown is a histological section of
25  osteotomy gap tissue from the control animal used in FIG
9A.   The section is characterized by the presence of
granulation tissue fibroblasts and capillaries.

           FIG. 10.   PLJ-HPTH1-34 expression construct.  A cDNA
30  fragment coding for a prepro-hPTH1-34 peptide was
generated by PCR™ (Hendy et al., 1981) and then ligated
into a BamHI cloning site in the PLJ retroviral
expression vector (Wilson et al., 1992).  Several
independent clones with the insert in the coding
35  orientation were isolated and characterized.

           FIG. 11.   Southern analysis of retroviral

integration in the YZ-15 clone.  10 mg of YZ-15 genomic
DNA were digested with *KpnI* (for which there is a unique
site in the vector LTR) and analyzed by Southern
blotting.  A cDNA fragment that coded for prepro-hPTH1-35

5    was used as a probe.  The positive control for the
Southern hybridization conditions was a *KpnI* digest of
genomic DNA from Rat-1 cells infected and selected with
the recombinant, replication-defective retrovirus PLJ-
hPTH1-84 (Wilson *et al.*, 1992).  *KpnI* digests of DNA were

10   also prepared from two negative controls:  native Rat-1
cells and Rat-1 cells infected and selected with BAG
("BAG cells", (Wilson *et al.*, 1992), a replication-
defective recombinant retrovirus that encodes $\beta$-
galactosidase, which is an irrelevant marker gene in

15   these studies.  Lane assignments were as follows:  1,
PLJ-hPTH1-84 cells; 2 BAG cells; 3, YZ-15; 4, native Rat-
1 cells.  DNA sizes (kb) are shown at the left of the
figure.  As expected, a fragment of the predicted size
(e.g., 4.3 kb) is seen only in lane 1 (the positive

20   control) and in lane 3 (YZ-15 DNA).


     FIG. 12.  Northern blot analysis of a transduced
Rat-1 clone.  Poly-A$(^+)$RNA was prepared from the YZ-15
clone and analyzed by Northern blotting as described

25   (Chen *et al.*, 1993).  FIG. 12 contains two panels on a
single sheet.  Poly-A$(^+)$ RNA prepared from PLJ-hPTH1-84
cells, BAG cells, and native Rat-1 cells were used as
positive and negative controls.  Four probes were applied
to a single blot following sequential stripping:  hPTH1-

30   34, $\beta$-gal, *Neo*, and $\beta$-actin.  Lane assignments were as
follows:  1, PLJ-hPTH1-84 cells; 2, BAG cells; 3, YZ-15
cells; 4, native Rat-1 cells.  As expected, the hPTH1-34
transcript is seen only in lane 1 (positive control) and
in lane 3-4; a *Neo* transcript is seen in lanes 1-3; a

35   $\beta$-gal transcript is seen only in lane 2; and $\beta$-actin
transcripts are seen in lanes 1-4.

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FIG. 13. Northern analysis of poly-A(⁺) RNA
demonstrating expression of the PTH/PTHrP receptor in
osteotomy repair tissue.

5        FIG. 14. Overlapping murine cDNA clones
representing the LTBP-like (LTBP-3) sequence. A partial
representation of restriction sites is shown. N, *NcoI*;
P, *PvuII*; R, *RsaII*; B, *BamHI*; H, *HindIII*. The numbering
system at the bottom assumes that the "A" of the
10       initiator Met codon is nt #1.

         FIG. 15A. A schematic showing the structure of the
murine fibrillin-1 gene product. Structural domains are
shown below the diagram. Symbols designating various
15       structural elements are defined in the legend to FIG.
15B.

         FIG. 15B. A schematic showing the structure of the
murine LTBP-like (LTBP-3) molecule. Domains #1-5 are
20       denoted below the diagram. Symbols designate the
following structural elements: EGF-CB repeats: open
rectangles; TGF-bp repeats: open ovals; Fib motif: open
circle; TGF-bp-like repeat: patterned oval; cysteine-rich
sequences: patterned rectangles; proline/glycine-rich
25       region: thick curved line, domain #2; proline-rich
region, thick curved line, domain #3. Note that symbols
designating the signal peptide have been deleted for
simplicity. Additionally, the schematic assumes that
EGF-like and EGF-CB repeats may extend for several amino
30       acids beyond the $C_6$ position.

         FIG. 15C. A schematic showing the structure of
human LTBP-1. Domains #1-5 are denoted below the
diagram. The symbols designating the structural elements
35       are defined in the legend to FIG. 15B.

         FIG. 16. Overview of expression of the new LTBP-

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like (LTBP-3) gene during murine development as
determined by tissue *in situ* hybridization.  FIG. 16
consists of autoradiograms made by direct exposure of
tissue sections to film after hybridization with
5    radiolabeled probes.  Day 8.5-9.0 sections contained
embryos surrounded by intact membranes, uterine tissues,
and the placental disk, cut in random planes.  Day 13.5
and 16.5 sections contain isolated whole embryos
sectioned in the sagittal plane near or about the mid-
10   line.  Identical conditions were maintained throughout
autoradiography and photography, thereby allowing a
comparison of the overall strength of hybridization in
all tissue sections.  The transcript is expressed in
connective tissue, mesenchyme, liver, heart and CNS.
15

**FIG. 17A.**  Selected microscopic views of mouse
LTBP-3 gene expression in day 8.5-9.0 p.c. mouse
developing tissues.  All photographs in FIG. 17A- FIG.
17D were taken from the same slides used to prepare whole
20   mount sections (after dipping slides in radiographic
emulsion).  Shown is the neural tube, brightfield image.
1 cm = 20 mm.

**FIG. 17B.**  Selected microscopic views of mouse
25   LTBP-3 gene expression in day 8.5-9.0 p.c. mouse
developing tissues.  Shown is the neural tube, darkfield
image. Note expression by neuroepithelial cells and by
surrounding mesenchyme. 1 cm = 20 mm.

30   **FIG. 17C.**  Selected microscopic views of mouse
LTBP-3 gene expression in day 8.5-9.0 p.c. mouse
developing tissues.  Shown is the heart, brightfield
image.  The figure demonstrates expression by myocardial
and endocardial (arrowheads) cells.  1 cm = 20 mm.
35

**FIG. 17D.**  Selected microscopic views of mouse
LTBP-3 gene expression in day 8.5-9.0 p.c. mouse

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developing tissues.  Shown is the heart, darkfield image.
The figure demonstrates expression by myocardial and
endocardial (arrowheads) cells. ·Darkfield
photomicrographs were taken after exposure of tissues to
5    photographic emulsion for 2 weeks.   In this image and
the one shown in FIG. 17B, red blood cell and other
plasma membranes give a faint white signal that
contributes to the background of the experiment.  1 cm =
20 mm.
10

    FIG. 18A.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
tissues.  All photographs in FIG. 18A - FIG. 18P were
taken from the same slides used to prepare whole mount
15    sections (after dipping slides in radiographic emulsion).
Shown is the cartilage model of developing long bone from
lower extremity, brightfield image.  Expression by
chondrocytes and by perichondrial cells is seen in FIG.
18B.  1 cm = 20 mm.
20

    FIG. 18B.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
tissues.  Shown is the cartilage model of developing long
bone from lower extremity, darkfield image.  Note
25    expression by chondrocytes and by perichondrial cells.
In all darkfield views of FIG. 18, red blood cell and
other plasma membranes give a faint white signal that
contributes to the background of the experiment.  Note
the absence of spurious hybridization signal in areas of
30    the slide that lack cellular elements.  1 cm = 20 mm.

    FIG. 18C.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
tissues.  Shown is the lung, brightfield image.  1 cm =
35    20 mm.

    FIG. 18D.  Microscopy of mouse LTBP-3 gene

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expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, brightfield image. 1 cm = 20 mm.

5        FIG. 18E.  Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, darkfield image. Note expression by epithelial cells of developing airway and by the surrounding parenchymal cells. 1 cm = 20 mm.

10        FIG. 18F.  Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, darkfield image. Note continuing expression by myocardial cells. 1 cm = 20 mm.

15        FIG. 18G.  Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, brightfield image. 1 cm = 20 mm.

20        FIG. 18H.  Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the intestine, brightfield image. 1 cm = 20 mm.

25        FIG. 18I.  Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, darkfield image. Note expression by acinar epithelial cells. 1 cm = 20 mm.

30        FIG. 18J.  Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is intestine, darkfield image. Note the expression in epithelial and subepithelial cells. 1 cm = 20 mm.

35

        FIG. 18K.  Microscopy of mouse LTBP-3 gene

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expression in day 13.5 and day 16 p.c. mouse developing
tissues.  Shown is kidney, brightfield image.  1 cm = 20
mm.

5          FIG. 18L.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
tissues. ·Shown is skin, brightfield image.  1 cm = 20
mm.

10          FIG. 18M.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
tissues.  Shown is kidney, darkfield image.  Note
expression by blastemal cells beneath the  kidney
capsule, epithelial cells of developing nephrons and
15    tubules, and the interstitial mesenchyme. 1 cm = 20 mm.

          FIG. 18N.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
tissues.  Shown is the skin, darkfield image.  Note the
20    expression by epidermal, adnexal and dermal cells of
developing skin. 1 cm = 20 mm.

          FIG. 18O.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
25    tissues. Shown is the retina, brightfield image.  1 cm =
20 mm.

          FIG. 18P.  Microscopy of mouse LTBP-3 gene
expression in day 13.5 and day 16 p.c. mouse developing
30    tissues.  Shown is the retina, darkfield image.  Note
expression by retinal epithelial cells and by adjacent
connective tissue cells. 1 cm = 20 mm.

          FIG. 19.  Time-dependent expression of the LTBP-3
35    gene by MC3T3-E1 cells.  mRNA preparation and Northern
blotting were preformed as described in Example XIV.
Equal aliquots of total RNA as determined by UV

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spectroscopy were loaded in each lane of the Northern
gel. As demonstrated by UV spectroscopy were loaded in
each lane of the Northern gel. As demonstrated by
methylene blue staining (Sambrook et al., 1989), equal
5    amounts of RNA were transferred to the nylon membrane.
The results demonstrate a clear, strong peak in LTBP-3
gene expression by 14 days in culture. Weaker signals
denoting LTBP-3 gene expression also can be observed
after 5 days and 28 days in culture.
10

    FIG. 20. Antisera #274 specifically binds LTBP-3
epitopes. Transfection of 293T cells with a full length
mouse LTBP-3 expression plasmid followed by
radiolabeling, preparation of medium sample,
15   immunoprecipitation, and 4-18% gradient SDS-PAGE were
performed as described in Example XIV. The figure
presents a SDS-PAGE autoradiogram of medium samples
following a 2 day exposure to film. Lane assignments are
as follows: Lane 1, radiolabeled 293T medium (prior to
20   transfection) immunoprecipitated with preimmune serum;
Land 2, radiolabeled 293T medium (prior to transfection)
immunoprecipitated with antibody #274; Lane 3,
radiolabeled 393T medium (following transfection and
preincubation with 10 μg of LTBP-3 synthetic peptide
25   cocktail) immunoprecipitated with antibody #274; and Lane
4, radiolabeled 293T medium (following transfection)
immunoprecipitated with antibody #274. As indicated by
the bar, the full length LTBP-3 molecule migrated at
180-190 kDa.
30

    FIG. 21. Co-immunoprecipitation of LTBP-3 and
TGR-$\beta$1 produced by MC3T3-E1 cells. Aliquots (~$10^6$
incorporated CPM) of radiolabeled media produced by
MC3T3-E1 cells after 7 days in culture were
35   immunoprecipitated as described in Example XIV. Bars
indicate the position of cold molecular weight standards
used to estimate molecular weight (Rainbow mix,

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Amersham). Immunoprecipitates were separated using
4%-18% gradient SDS-PAGE and reducing conditions. The
figure shows a negative control lane 1 consisting of
MC3T3-E1 medium immunoprecipitated with anti-LTBP-3
5   antibody #274. Western blotting was performed using the
lower portion of the gradient gel and a commercially
available antibody to TGF-$\beta$1 (Santa Cruz Biotechnology,
Inc.). Antibody staining was detected using commercially
available reagents and protocols (ECL Western Blotting
10  Reagent, Amersham). MC3T3-E1 medium was
immunoprecipitated with anti-LTBP-2 antibody #274.

      **FIG. 22A.** Radiographic analysis of the type II
collagen osteotomy gap three weeks after surgery.
15

      **FIG. 22B.** Radiographic analysis of the type I
collagen osteotomy gap three weeks after surgery.

      **FIG. 22C.** Histologic analysis of the type II
20  collagen osteotomy shown in FIG. 22A.

      **FIG. 23A.** Adenovirus-mediated gene transfer into
bone repair/regeneration cells *in vivo*. Positive
(arrows) $\beta$-gal cytoplasmic staining is observed in the
25  fracture repair cells.

      **FIG. 23B.** Adenovirus-mediated gene transfer into
bone repair/regeneration cells *in vivo*. Serial section
negative control strained with the vehicle of the $\beta$-gal
30  antibody plus a cocktail of non-specific rabbit IgG
antibodies.

      **FIG. 23C.** Adenovirus-mediated gene transfer into
bone repair/regeneration cells *in vivo*. Osteotomy site
35  was filled with a fibrous collagen implant material
soaked in a solution of the replication-defective
recombinant adenovirus AdRSV$\beta$-gal ($\sim$10$^{11}$ plaque forming

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units/ml). Note the positive (arrow) β-gal nuclear
staining of chondrocytes within the osteotomy site, as
demonstrated by immunohistochemistry using a specific
anti-β-gal antibody.

5

FIG. 24. The murine BMP-4 amino acid sequence, SEQ
ID NO:1. The HA epitope is shown in bold at the extreme
carboxy terminus of the sequence.

10    FIG. 25. DNA sequence of the murine LTBP-3 gene
(SEQ ID NO:2).

FIG. 26. Amino acid sequence of the murine LTBP-3
gene product (SEQ ID NO:3).

15

FIG. 27. DNA sequence of the murine LTBP-2 gene
(SEQ ID NO:17).

FIG. 28. Amino acid sequence of the murine LTBP-2
20    gene product (SEQ ID NO:18).


## DESCRIPTION OF THE PREFERRED EMBODIMENT

25    1.    Applications of Bone Repair Technology to Human
           Treatment

The following is a brief discussion of four human
conditions to exemplify the variety of diseases and
30    disorders that would benefit from the development of new
technology to improve bone repair and healing processes.
In addition to the following, several other conditions,
such as, for example, vitamin D deficiency; wound healing
in general; soft skeletal tissue repair; and cartilage
35    and tendon repair and regeneration, may also benefit from
technology concerning the stimulation of bone progenitor
cells.

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The first example is the otherwise healthy
individual who suffers a fracture. Often, clinical bone
fracture is treated by casting to alleviate pain and
allow natural repair mechanisms to repair the wound.
5   While there has been progress in the treatment of
fracture in recent times, even without considering the
various complications that may arise in treating
fractured bones, any new procedures to increase bone
healing in normal circumstances would represent a great
10  advance.

A second example which may benefit from new
treatment methods is osteogenesis imperfecta (OI). OI
encompasses various inherited connective tissue diseases
15  that involve bone and soft connective tissue fragility in
humans (Byers and Steiner, 1992; Prockop, 1990). About
one child per 5,000-20,000 born is affected with OI and
the disease is associated with significant morbidity
throughout life. A certain number of deaths also occur,
20  resulting in part from the high propensity for bone
fracture and the deformation of abnormal bone after
fracture repair (OI types II-IV; Bonadio and Goldstein,
1993). The relevant issue here is quality of life;
clearly, the lives of affected individuals would be
25  improved by the development of new therapies designed to
stimulate and strengthen the fracture repair process.

OI type I is a mild disorder characterized by bone
fracture without deformity, blue sclerae, normal or near
30  normal stature, and autosomal dominant inheritance
(Bonadio and Goldstein, 1993). Osteopenia is associated
with an increased rate of lone bone fracture upon
ambulation (the fracture frequency decreases dramatically
at puberty and during young adult life, but increases
35  once again in late middle age). Hearing loss, which
often begins in the second or third decade, is a feature
of this disease in about half the families and can

progress despite the general decline in fracture
frequency. Dentinogenesis imperfecta is observed in a
subset of individuals.

5    In contrast, OI types II-VI represent a spectrum of
more severe disorders associated with a shortened life-
span. OI type II, the perinatal lethal form, is
characterized by short stature, a soft calvarium, blue
sclerae, fragile skin, a small chest, floppy appearing
10   lower extremities (due to external rotation and abduction
of the femurs), fragile tendons and ligaments, bone
fracture with severe deformity, and death in the
perinatal period due to respiratory insufficiency.
Radiographic signs of bone weakness include compression
15   of the femurs, bowing of the tibiae, broad and beaded
ribs, and calvarial thinning.

OI type III is characterized by short stature, a
triangular facies, severe scoliosis, and bone fracture
20   with moderate deformity. Scoliosis can lead to emphysema
and a shortened life-span due to respiratory
insufficiency. OI type IV is characterized by normal
sclerae, bone fracture with mild to moderate deformity,
tooth defects, and a natural history that essentially is
25   intermediate between OI type II and OI type I.

More than 200 OI mutations have been characterized
since 1989 (reviewed in Byers and Steiner, 1992; Prockop,
1990). The vast majority occur in the COL1A1 and COL1A2
30   genes of type I collagen. Most cases of OI type I appear
to result from heterozygous mutations in the COL1A1 gene
that decrease collagen production but do not alter
primary structure, i.e., heterozygous null mutations
affecting COL1A1 expression. Most cases of OI types II-
35   IV result from heterozygous mutations in the COL1A1 and
COL1A2 genes that alter the structure of collagen.

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A third important example is osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. An estimated 20-25 million people are at increased risk for
5    fracture because of site-specific bone loss. Risk factors for osteoporosis include increasing age, gender (more females), low bone mass, early menopause, race (Caucasians), low calcium intake, reduced physical activity, genetic factors, environmental factors
10   (including cigarette smoking and abuse of alcohol or caffeine), and deficiencies in neuromuscular control that create a propensity to fall.

More than a million fractures in the USA each year
15   can be attributed to osteoporosis, and in 1986 alone the treatment of osteoporosis cost an estimated 7-10 billion health care dollars. Demographic trends (i.e., the gradually increasing age of the US population) suggest that these costs may increase 2-3 fold by the year 2020
20   if a safe and effective treatment is not found. Clearly, osteoporosis is a significant health care problem.

Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in
25   middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age. Much of the morbidity and mortality associated with osteoporosis results from immobilization of elderly patients following fracture.
30

Current therapies for osteoporosis patients focus on fracture prevention, not fracture repair. This remains an important consideration because of the literature, which clearly states that significant morbidity and
35   mortality are associated with prolonged bed rest in the elderly, particularly those who have suffered hip fractures. Complications of bed rest include blood clots

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and pneumonia. These complications are recognized and
measures are usually taken to avoid them, but these
measures hardly represent the best approach to therapy.
Thus, the osteoporotic patient population would benefit
5      from new therapies designed to strengthen bone and speed
up the fracture repair process, thereby getting these
people on their feet before the complications arise.

A fourth example is related to bone reconstruction
10     and, specifically, the ability to reconstruct defects in
bone tissue that result from traumatic injury; cancer or
cancer surgery; birth defect; a developmental error or
heritable disorder; or aging. There is a significant
orthopaedic need for more stable total joint implants,
15     and cranial and facial bone are particular targets for
this type of reconstructive need. The availability of
new implant materials, e.g., titanium, has permitted the
repair of relatively large defects. Titanium implants
provide excellent temporary stability across bony
20     defects. However, experience has shown that a lack of
viable bone bridging the defect can result in exposure of
the appliance, infection, structural instability and,
ultimately, failure to repair the defect.

25     Autologous bone grafts are another possible
reconstructive modality, but they have several
demonstrated disadvantages in that they must be harvested
from a donor site such as iliac crest or rib, they
usually provide insufficient bone to completely fill the
30     defect, and the bone that does form is sometimes prone to
infection and resorption. Partially purified xenogeneic
preparations are not practical for clinical use because
microgram quantities are purified from kilograms of
bovine bone, making large scale commercial production
35     both costly and impractical. Allografts and
demineralized bone preparations are therefore often
employed.

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Microsurgical transfers of free bone grafts with attached soft tissue and blood vessels can close bony defects with an immediate source of blood supply to the graft. However, these techniques are time consuming,

5    have been shown to produce a great deal of morbidity, and can only be used by specially trained individuals. Furthermore, the bone implant is often limited in quantity and is not readily contoured. In the mandible, for example, the majority of patients cannot wear dental

10   appliances using presently accepted techniques (even after continuity is established), and thus gain little improvement in the ability to masticate. Toriumi et al., have written that, "reconstructive surgeons should have at their disposal a bone substitute that would be

15   reliable, biocompatible, easy to use, and long lasting and that would restore mandibular continuity with little associated morbidity."

In connection with bone reconstruction, specific

20   problem areas for improvement are those concerned with treating large defects, such as created by trauma, birth defects, or particularly, following tumor resection. The success of orthopaedic implants, interfaces and artificial joints could conceivably be improved if the

25   surface of the implant, or a functional part of an implant, were to be coated with a bone stimulatory agent. The surface of implants could be coated with one or more appropriate materials in order to promote a more effective interaction with the biological site

30   surrounding the implant and, ideally, to promote tissue repair.

**2.   Bone Repair**

35       Bone tissue is known to have the capacity for repair and regeneration and there is a certain understanding of the cellular and molecular basis of these processes. The

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initiation of new bone formation involves the commitment,
clonal expansion, and differentiation of progenitor
cells.  Once initiated, bone formation is promoted by a
variety of polypeptide growth factors, Newly formed bone
5    is then maintained by a series of local and systemic
growth and differentiation factors.

The concept of specific bone growth-promoting agents
is derived from the work of Huggins and Urist.  Huggins
10   et al., 1936, demonstrated that autologous
transplantation of canine incisor tooth to skeletal
muscle resulted in local new bone formation (Huggins et
al., 1936).  Urist and colleagues reported that
demineralized lyophilized bone segments induced bone
15   formation (Urist, 1965; Urist et al., 1983), a process
that involved macrophage chemotaxis; the recruitment of
progenitor cells; the formation of granulation tissue,
cartilage, and bone; bone remodeling; and marrow
differentiation.  The initiation of cartilage and bone
20   formation in an extraskeletal site, a process referred to
as osteoinduction, has permitted the unequivocal
identification of initiators of bone morphogenesis
(Urist, 1965; Urist et al., 1983; Sampath et al., 1984;
Wang et al., 1990; Cunningham et al., 1992).
25

Significant progress has now been made in
characterizing the biological agents elaborated by active
bone tissue during growth and natural bone healing.
Demineralized bone matrix is highly insoluble; Sampath
30   and Reddi (1981) showed that only 3% of the proteins can
be extracted using strong combinations of denaturants and
detergents.  They also showed that the unfractionated
demineralized bone extract will initiate bone
morphogenesis, a critical observation that led to the
35   purification of "osteoinductive" molecules.  Families of
proteinaceous osteoinductive factors have now been
purified and characterized.  They have been variously

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referred to in the literature as bone morphogenetic or
morphogenic proteins (BMPs), osteogenic bone inductive
proteins or osteogenic proteins (OPs).

5    3.    **Bone Repair and Bone Morphogenetic Proteins (BMPs)**

Following their initial purification, several bone
morphogenetic protein genes have now been cloned using
molecular techniques (Wozney et al., 1988; Rosen et al.,
10   1989; summarized in Alper, 1994). This work has
established BMPs as members of the transforming growth
factor-$\beta$ (TGF-$\beta$) superfamily based on DNA sequence
homologies. Other TGF molecules have also been shown to
participate in new bone formation, and TGF-$\beta$ is regarded
15   as a complex multifunctional regulator of osteoblast
function (Centrella et al., 1988; Carrington et al.,
1988; Seitz et al., 1992). Indeed, the family of
transforming growth factors (TGF-$\beta$1, TGF-$\beta$2, and TGF-$\beta$3)
has been proposed as potentially useful in the treatment
20   of bone disease (U.S. Patent 5,125,978, incorporated
herein by reference).

The cloning of distinct BMP genes has led to the
designation of individual BMP genes and proteins as BMP-1
25   through BMP-8. BMPs 2-8 are generally thought to be
osteogenic (BMP-1 may be a more generalized morphogen;
Shimell et al., 1991). BMP-3 is also called osteogenin
(Luyten et al., 1989) and BMP-7 is also called OP-1
(Ozkaynak et al., 1990). TGFs and BMPs each act on cells
30   via complex, tissue-specific interactions with families
of cell surface receptors (Roberts and Sporn, 1989;
Paralkar et al., 1991).

Several BMP (or OP) nucleotide sequences and
35   vectors, cultured host cells and polypeptides have been
described in the patent literature. For example, U.S.
Patents, 4,877,864, 4,968,590 and 5,108,753 all concern

osteogenic factors.  More specifically, BMP-1 is
disclosed in U.S. Patent 5,108,922; BMP-2 species,
including MBP-2A and BMP-2B, are disclosed in U.S.
Patents 5,166,058, 5,013,649, and 5,013,649; BMP-3 in

5      5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; and
BMP-7 in 5,108,753 and 5,141,905; all incorporated herein
by reference.  Various BMP clones and their activities
are particularly described by Wozney et al., (1988;
incorporated herein by reference).  DNA sequences

10     encoding the osteogenic proteins designated OP-1, COP-5
and COP-7 are also disclosed in U.S. Patent 5,011,691.
Although the BMP terminology is widely used, it may prove
to be the case that there is an OP counterpart term for
every individual BMP (Alper, 1994).

15

**4.    Bone Repair and Growth Factors and Cytokines**

       Transforming growth factors (TGFs) have a central
role in regulating tissue healing by affecting cell
20     proliferation, gene expression, and matrix protein
synthesis (Roberts and Sporn, 1989).  While not
necessarily a direct effect, Bolander and colleagues have
provided evidence that TGF-$\beta$1 and TGF-$\beta$2 can initiate
both chondrogenesis and osteogenesis (Joyce et al., 1990;
25     Izumi et al., 1992; Jingushi et al., 1992).  In these
studies new cartilage and bone formation appeared to be
dose dependent (i.e., dependent on the local growth
factor concentration).  The data also suggested that TGF-
$\beta$1 and TGF-$\beta$2 stimulated cell differentiation by a
30     similar mechanism, even though they differed in terms of
the ultimate amount of new cartilage and bone that was
formed.

       Other growth factors/hormones besides TGF and BMP
35     may influence new bone formation following fracture.
Bolander and colleagues injected recombinant acidic
fibroblast growth factor into a rat fracture site

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(Jingushi *et al.*, 1990). The major effect of multiple
high doses (1.0 mg/50 ml) was a significant increase in
cartilage tissue in the fracture gap, while lower doses
had no effect. These investigators also used the reverse
5    transcriptase-polymerase chain reaction (PCR™) technique
to demonstrate expression of estrogen receptor
transcripts in callus tissue (Boden *et al.*, 1989). These
results suggested a role for estrogen in normal fracture
repair.
10

Horowitz and colleagues have shown that activated
osteoblasts will synthesize the cytokine, macrophage
colony stimulating factor (Horowitz *et al.*, 1989). The
osteotropic agents used in this study included
15    lipopolysaccharide, PTH1-84, PTH1-34, vitamin D and
all-trans retinoic acid. This observation has led to the
suggestion that osteoblast activation following fracture
may lead to the production of cytokines that regulate
both hematopoiesis and new bone formation. Various other
20    proteins and polypeptides that have been found to be
expressed at high levels in osteogenic cells, such as,
e.g., the polypeptide designated Vgr-1 (Lyons *et al.*,
1989), also have potential for use in connection with the
present invention.
25

## 5.   Bone Repair and Calcium Regulating Hormones

Calcium regulating hormones such as parathyroid
hormone (PTH) participate in new bone formation and bone
30    remodeling (Raisz and Kream, 1983). PTH is an 84 amino
acid calcium-regulating hormone whose principle function
is to raise the $Ca^{+2}$ concentration in plasma and
extracellular fluid. Studies with the native hormone and
with synthetic peptides have demonstrated that the amino-
35    terminus of the molecule (aa 1-34) contains the
structural requirements for biological activity (Tregear
*et al.*, 1973; Hermann-Erlee *et al.*, 1976; Riond, 1993).

PTH functions by binding to a specific cell surface
receptor that belongs to the G protein-coupled receptor
superfamily (Silve *et al.*, 1982; Rizzoli *et al.*, 1983;
Juppner *et al.*, 1991).

5

Using a retroviral approach, a human full-length PTH
gene construct has been introduced into cultured rat
fibroblasts to create recombinant PTH-secreting cells.
These cells were then transplanted into syngeneic rat
10   recipients that were observed to develop hypercalcemia
mediated by the increased serum concentrations of PTH
(Wilson *et al.*, 1992). The object of these studies was
to create an animal model of primary hyperparathyroidism.

15   PTH has a dual effect on new bone formation, a
somewhat confusing aspect of hormone function despite
intensive investigation. PTH has been shown to be a
potent direct inhibitor of type I collagen production by
osteoblasts (Kream *et al.*, 1993). Intact PTH was also
20   shown to stimulate bone resorption in organ culture over
30 years ago, and the hormone is known to increase the
number and activity of osteoclasts. Recent studies by
Gay and colleagues have demonstrated binding of
[$^{125}$I]PTH(1-84) to osteoclasts in tissue sections and that
25   osteoclasts bind intact PTH in a manner that is both
saturable and time- and temperature dependent (Agarwala
and Gay, 1992). While these properties are consistent
with the presence of PTH/PTHrP receptors on the
osteoclast cell surface, this hypothesis is still
30   considered controversial. A more accepted view, perhaps,
is that osteoclast activation occurs via an osteoblast
signaling mechanism.

On the other hand, osteosclerosis may occur in human
35   patients with primary hyperparathyroidism (Seyle, 1932).
It is well known that individuals with
hyperparathyroidism do not inexorably lose bone mass, but

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eventually achieve a new bone remodeling steady state
after an initial period of net bone loss. Chronic, low
dose administration of the amino-terminal fragment of PTH
(aa 1-34) also can induce new bone formation according to
5    a time- and dose-dependent schedule (Seyle, 1932; Parsons
and Reit, 1974).

Human PTH1-34 has recently been shown to: stimulate
DNA synthesis in chick osteoblasts and chondrocytes in
10   culture (van der Plas, 1985; Schluter *et al.*, 1989;
Somjen *et al.*, 1990); increase bone cell number *in vivo*
(Malluche *et al.*, 1986); enhance the *in vitro* growth of
chick embryonic cartilage and bone (Kawashima, 1980;
Burch and Lebovitz, 1983; Lewinson and Silbermann, 1986;
15   Endo *et al.*, 1980; Klein-Nulend *et al.*, 1990); enhance
surface bone formation (both cortical and trabecular
bone) in normal and osteogenic animals and in humans with
osteoporosis (Reeve *et al.*, 1976; Reeve *et al.*, 1980; Tam
*et al.*, 1982; Hefti *et al.*, 1982; Podbesek *et al.*, 1983;
20   Stevenson and Parsons, 1983; Slovik *et al.*, 1986;
Gunness-Hey and Hock, 1984; Tada *et al.*, 1988; Spencer *et
al.*, 1989; Hock and Fonseca, 1990; Liu and Kalu, 1990;
Hock and Gera, 1992; Mitlak *et al.*, 1992; Ejersted *et
al.*, 1993); and delay and reverse the catabolic effects
25   of estrogen deprivation on bone mass (Hock *et al.*, 1988;
Hori *et al.*, 1988; Gunness-Hey and Hock, 1989; Liu *et
al.*, 1991). Evidence of synergistic interactions between
hPTH-1-34 and other anabolic molecules has been
presented, including insulin-like growth factor, BMP-2,
30   growth hormone, vitamin D, and TGF-$\beta$ (Slovik *et al.*,
1986; Spencer *et al.*, 1989; Mitlak *et al.*, 1992; Canalis
*et al.*, 1989; Linkhart and Mohan, 1989; Seitz *et al.*,
1992; Vukicevic *et al.*, 1989).

35   Anecdotal observation has shown that serum PTH
levels may be elevated following bone fracture (Meller *et
al.*, 1984; Johnston *et al.*, 1985; Compston *et al.*, 1989;

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Hardy *et al.*, 1993), but the significance of this
observation is not understood.  There are apparently no
reports in the literature concerning attempts to localize
either PTH or the PTH/PTHrP receptor *in situ* in human

5    fracture sites or in experimental models.  Furthermore,
no attempt has been made to augment bone repair by the
exogenous addition of PTH peptides.  Although hPTH1-34 is
known to function as an anabolic agent for bone, prior to
the present invention, much remained to be learned about

10   the role (if any) of PTH during bone regeneration and
repair.


**6.    Protein Administration and Bone Repair**


15       Several studies have been conducted in which
preparations of protein growth factors, including BMPs,
have been administered to animals in an effort to
stimulate bone growth.  The results of four such
exemplary studies are described blow.

20

         Toriumi *et al.*, studied the effect of recombinant
BMP-2 on the repair of surgically created defects in the
mandible of adult dogs (Toriumi *et al.*, 1991).  Twenty-
six adult hounds were segregated into three groups

25   following the creation of a 3 cm full thickness
mandibular defect:  12 animals received test implants
composed of inactive dog bone matrix carrier and human
BMP-2, 10 animals received control implants composed of
carrier without BMP-2, and BMP-4 animals received no

30   implant.  The dogs were euthanized at 2.5-6 months, and
the reconstructed segments were analyzed by radiography,
histology, histomorphometry, and biomechanical testing.
Animals that received test implants were euthanized after
2.5 months because of the presence of well mineralized

35   bone bridging the defect.  The new bone allowed these
animals to chew a solid diet, and the average bending
strength of reconstructed mandibles was 27% of normal

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('normal' in this case represents the unoperated,
contralateral hemimandible). In contrast, the implants
in the other two groups were non-functional even after
6 months and showed minimal bone formation.

5

Yasko et al., published a related study in which the
effect of BMP-2 on the repair of segmental defects in the
rat femur was examined (Yasko et al., 1992). The study
design included a group that received a dose of 1.4 mg of
10    BMP-2, another group that received 11.0 mg of BMP-2, and
a control group that received carrier matrix alone.
Endochondral bone formation was observed in both groups
of animals that received BMP-2. As demonstrated by
radiography, histology, and whole bone (torsion) tests of
15    mechanical integrity, the larger dose resulted in
functional repair of the 5-mm defect beginning 4.5 weeks
after surgery. The lower dose resulted in radiographic
and histological evidence of new bone formation, but
functional union was not observed even after 9 weeks post
20    surgery. There was also no evidence of bone formation in
control animals at this time.

Chen et al., showed that a single application of 25-
100 mg of recombinant TGF-$\beta$1 adjacent to cartilage
25    induced endochondral bone formation in the rabbit ear
full thickness skin wounds (Chen et al., 1991). Bone
formation began 21 days following the creation of the
wound and reached a peak at day 42, as demonstrated by
morphological methods. Active bone remodeling was
30    observed beyond this point.

In a related study, Beck et al., demonstrated that a
single application of TGF-$\beta$1 in a 3% methylcellulose gel
was able to repair surgically induced large skull defects
35    that otherwise heal by fibrous connective tissue and
never form bone (Beck et al., 1991). Bony closure was
achieved within 28 days of the application of 200 mg of

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TGF-β1 and the rate of healing was shown to be dose
dependent.

5      Studies such as those described above have thus
established that exogenous growth factors can be used to
stimulate new bone formation/repair/regeneration *in vivo*.
Certain U.S. Patents also concern methods for treating
bone defects or inducing bone formation.  For example,
U.S. Patent 4,877,864 relates to the administration of a
10     therapeutic composition of bone inductive protein to
treat cartilage and/or bone defects; U.S. Patent
5,108,753 concerns the use of a device containing a pure
osteogenic protein to induce endochondral bone formation
and for use in periodontal, dental or craniofacial
15     reconstructive procedures.

However, nowhere in this extensive literature does
there appear to be any suggestion that osteogenic genes
themselves may be applied to an animal in order to
20     promote bone repair or regeneration.  Indeed, even
throughout the patent literature that concerns genes
encoding various bone stimulatory factors and their *in
vitro* expression in host cells to produce recombinant
proteins, there seems to be no mention of the possibility
25     of using nucleic acid transfer in an effort to express an
osteogenic gene in bone progenitor cells *in vivo* or to
promote new bone formation in an animal or human subject.

7.   Biocompatible Matrices for use in Bone Repair
30

There is a considerable amount of work that has been
directed to the development of biocompatible matrices for
use in medical implants, including those specifically for
bone implantation work.  In context of the present
35     invention, a matrix may be employed in association with
the gene or DNA coding region encoding the osteotropic
polypeptide in order to easily deliver the gene to the

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site of bone damage. Such matrices may be formed from a
variety of materials presently in use for implanted
medical applications.

5       In certain cases, the matrix may also act as a
"biofiller" to provide a structure for the developing
bone and cartilage. However, the formation of such a
scaffolding structure is not a primary requirement,
rather, the main requirements of the matrix are to be
10      biocompatible and to be capable of delivering a nucleic
acid segment to a bone cell or bone tissue site.

Matrices that may be used in certain embodiments
include non-biodegradable and chemically defined
15      matrices, such as sintered hydroxyapatite, bioglass,
aluminates, and other ceramics. The bioceramics may be
altered in composition, such as in calcium-aluminate-
phosphate; and they may be processed to modify particular
physical and chemical characteristics, such as pore size,
20      particle size, particle shape, and biodegradability.
Certain polymeric matrices may also be employed if
desired, these include acrylic ester polymers and lactic
acid polymers, as disclosed in U.S. Patents 4,526,909,
and 4,563,489, respectively, each incorporated herein by
25      reference. Particular examples of useful polymers are
those of orthoesters, anhydrides, propylene-cofumarates,
or a polymer of one or more $\alpha$-hydroxy carboxylic acid
monomers, e.g., $\alpha$-hydroxy acetic acid (glycolic acid)
and/or $\alpha$-hydroxy propionic acid (lactic acid).
30
Some of the preferred matrices for use in present
purposes are those that are capable of being resorbed
into the body. Potential biodegradable matrices for use
in bone gene transfer include, for example, PLGA block
35      copolymers, biodegradable and chemically defined calcium
sulfate, tricalciumphosphate, hydroxyapatite, and
polyanhydrides. Furthermore, biomatrices comprised of

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pure proteins and/or extracellular matrix components may
be employed.

The inventors have shown the use of bone or dermal
5       collagenous materials as matrices, as may be prepared
from various commercially-available lyophilized collagen
preparations, such as those from bovine or rat skin, as
well as PLGA block copolymers.  Collagen matrices may
also be formulated as described in U.S. Patent 4,394,370,
10      incorporated herein by reference, which concerns the use
of collagenous matrices as delivery vehicles for
osteogenic protein.  UltraFiber™, as may be obtained from
Norian Corp. (Mountain View, CA), is a preferred matrix.
Preferred matrices are those formulated with type II
15      collagen, and most preferably, recombinant type II
collagen and mineralized type II collagen.

Further suitable matrices may also be prepared from
combinations of materials, such as PLGA block copolymers,
20      which allow for sustained release; hydroxyapatite; or
collagen and tricalciumphosphate.  Although sufficient
sequestration and subsequent delivery of an osteotropic
gene is in no way a limitation of the present invention,
should it be desired, a porous matrix and gene
25      combination may also be administered to the bone tissue
site in combination with an autologous blood clot.  The
basis for this is that blood clots have previously been
employed to increase sequestration of osteogenic proteins
for use in bone treatment (U.S. Patent 5,171,579,
30      incorporated herein by reference) and their use in
connection with the present invention is by no means
excluded (they may even attract growth factors for
cytokines).

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8.  Collagen

Although not previously proposed for use with a
nucleic acid molecule, the use of collagen as a
pharmaceutical delivery vehicle has been described.  The
biocompatibility of collagen matrices is well known in
the art.  U.S. Patents 5,206,028, 5,128,136, 5,081,106,
4,585,797, 4,390,519, and 5,197,977 (all incorporated
herein by reference) describe the biocompatibility of
collagen-containing matrices in the treatment of skin
lesions, use as a wound dressing, and as a means of
controlling bleeding.  In light of these documents,
therefore, there is no question concerning the
suitability of applying a collagen preparation to a
tissue site of an animal.

U.S. Patent 5,197,977 describes the preparation of a
collagen-impregnated vascular graft including drug
materials complexed with the collagen to be released
slowly from the graft following implant.  U.S. Patent
4,538,603 is directed to an occlusive dressing useful for
treating skin lesions and a granular material capable of
interacting with wound exudate.  U.S. Patent 5,162,430
describes a pharmaceutically acceptable, non-immunogenic
composition comprising a telopeptide collagen chemically
conjugated to a synthetic hydrophilic polymer.

Further documents that one of skill in the art may
find useful include U. S. Patents 4,837,285, 4,703,108,
4,409,332, and 4,347,234, each incorporated herein by
reference.  These references describe the uses of
collagen as a non-immunogenic, biodegradable, and
bioresorbable binding agent.

The inventors contemplate that collagen from many
sources will be useful in the present invention.
Particularly useful are the amino acid sequences of type

II collagen.  Examples of type II collagen are well known
in the art.  For example, the amino acid sequences of
human .(Lee *et al*., 1989), rat (Michaelson *et al*., 1994),
and murine (Ortman *et al*., 1994) have been determined
5    (SEQ ID NO:10, SEQ ID NO::12, and SEQ ID NO:14,
respectively).

Although not previously known to be capable of
stimulating bone progenitor cells itself, type II
10   collagen is herein surprisingly shown to possess this
property, which thus gives rise to new possibilities for
clinical uses.

9.    **Nucleic Acid Delivery**
15

The transfer of nucleic acids to mammalian cells has
been proposed a method for treating certain diseases or
disorders.  Nucleic acid transfer or delivery is often
referred to as "gene therapy".  Initial efforts toward
20   postnatal (somatic) gene therapy relied on indirect means
of introducing genes into tissues, *e.g.*, target cells
were removed from the body, infected with viral vectors
carrying recombinant genes, and implanted into the body.
These type of techniques are generally referred to as *ex*
25   *vivo* treatment protocols.  Direct *in vivo* gene transfer
has recently been achieved with formulations of DNA
trapped in liposomes (Ledley *et al*., 1987); or in
proteoliposomes that contain viral envelope receptor
proteins (Nicolau *et al*., 1983); calcium phosphate-
30   coprecipitated DNA (Benvenisty and Reshef, 1986); and DNA
coupled to a polylysine-glycoprotein carrier complex
(Wu and Wu, 1988).  The use of recombinant replication-
defective viral vectors to infect target cells *in vivo*
has also been described (*e.g.*, Seeger *et al*., 1984).
35

In recent years, Wolff *et al*., demonstrated that
direct injection of purified preparations of DNA and RNA

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into murine skeletal muscle resulted in significant
reporter gene expression (Wolff et al., 1990). This was
an unexpected finding, and the mechanism of gene transfer
could not be defined. The authors speculated that muscle
5       cells may be particularly suited to take up and express
polynucleotides in vivo or that damage associated with
DNA injection may allow transfection to occur.

Wolff et al., suggested several potential
10      applications of the direct injection method, including
(a) the treatment of heritable disorders of muscle, (b)
the modification of non-muscle disorders through muscle
tissue expression of therapeutic transgenes, (c) vaccine
development, and (d) a reversible type of gene transfer,
15      in which DNA is administered much like a conventional
pharmaceutical treatment. In an elegant study Liu and
coworkers recently showed that the direct injection
method can be successfully applied to the problem of
influenza vaccine development (Ulmer et al., 1993).
20

The use of gene transfer to synoviocytes as a means
of treating arthritis has also been discussed (Bandara et
al., 1992; Roessler et al., 1993). The protocols
considered have included both the ex vivo treatment of
25      isolated synoviocytes and their re-introduction into the
animal and also direct gene transfer in which suitable
vectors are injected into the joint. The transfer of
marker genes into synoviocytes has already been
demonstrated using both retroviral and adenoviral
30      technology (Bandara et al., 1992; Roessler et al., 1993).

Despite the exclusive emphasis on protein treatment
by those working in the field of new bone growth, the
present inventors saw that there was great potential for
35      using nucleic acids themselves to promote bone
regeneration/repair in vivo. This provides for a more
sophisticated type of pharmaceutical delivery. In

addition to the ease and cost of preparing DNA, it was
also reasoned that using DNA transfer rather than peptide
transfer would provide many further advantages. For
example, DNA transfer allows for the expression or over-
5    expression of integral membrane receptors on the surface
of bone regeneration/repair cells, whereas this cannot be
done using peptide transfer because the latter (a priori)
is an extracellular manipulation. Importantly, DNA
transfer also allows for the expression of polypeptides
10   modified in a site-directed fashion with the very minimum
of additional work (i.e., straightforward molecular
biological manipulation without protein purification) as
well as sustained release of therapies delivered by an
injectable route.

15

The advantages of using DNA are also manifold
regarding the development of pharmaceutical products and
effective means of delivery. Here, important advantages
include the ability to prepare injectable formulations,
20   especially those compositions that exhibit reversible
thermal gelation, and the opportunity to combine such
injectables with imaging technology during delivery.
"Sustained release" is also an important advantage of
using DNA, in that the exogenously added DNA continues to
25   direct the production of a protein product following
incorporation into a cell. The use of certain matrix-DNA
compositions also allows for a more typical "sustained
release" phenomenon in that the operative release of DNA
from the matrix admixture can also be manipulated.

30

The inventors contemplated that both naked DNA and
viral-mediate DNA could be employed in an effort to
transfer genes to bone progenitor cells. In beginning to
study this, the most appropriate animal model had to be
35   employed, that is, one in which the possibilities of
using nucleic acids to promote bone repair could be
adequately tested in controlled studies.

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10.  Osteotomy Model

Prior to the present invention, three model systems were available for study in this area, including Mov13

5    mice, an animal model of OI. Unfortunately, each of the models suffers from significant drawbacks. With the Mov13 mice, first, these mice typically die in young adulthood because of retrovirus-induced leukemia (Schnieke et al., 1983); second, gene transfer studies in

10   Mov13 mice conducted between postnatal weeks 8-16 (i.e., prior to the development of leukemia) may be complicated by a natural adaptation in which a significant amount of new bone is deposited on the periosteal surface (Bonadio et al., 1993); and third, an osteotropic gene transferred

15   into an osteotomy site may synergize with the active retrovirus and make it even more virulent.

Another system is the in vivo bone fracture model created by Einhorn and colleagues (Bonnarens and Einhorn,

20   1984). However, this model is a closed system that would not easily permit initial studies of gene transfer in vivo. The organ culture model developed by Bolander and colleagues (Joyce et al., 1990) was also available, but again, this model is not suitable for studying gene

25   transfer in vivo. Due to the unsuitability of the above models for studying the effects of gene transfer on bone repair and regeneration, the inventors employed a rat osteotomy system, as described below.

30   The important features of the rat osteotomy model are as follows: under general anesthesia, four 1.2 mm diameter pins are screwed into the femoral diaphysis of normal adult Sprague-Dawley rats. A surgical template ensures parallel placement of the pins. An external

35   fixator is then secured on the pins, and a 2 mm, or 5 mm, segmental defect is created in the central diaphysis with a Hall micro 100 oscillating saw. A biodegradable

implant material, soaked in a solution of plasmid DNA, other genetic construct or recombinant virus preparation, is then placed in the intramedullary canal and the defect is closed (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C,

5      FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

New bone formation can be detected as early as three weeks later in the 2 mm gap, although up to 9 weeks is generally allowed for new bone formation to occur. The

10     fixator provided the necessary stability, and there were no limitations on animal ambulation. The surgical protocol has been successfully performed on 21/21 animals to date. None of these animals have died. Assays of new bone formation are performed after sacrifice, except

15     plain film radiography, which is performed weekly from the time of surgery to sacrifice.

Previous studies in Sprague-Dawley rats have shown that the 5 mm osteotomy gap will heal as a fibrous non-

20     union, whereas a gap of less than 3 mm, (such as the 2 mm gap routinely employed in the studies described herein) will heal by primary bone formation. Studies using the 5 mm gap thus allow a determination of whether transgene expression can stimulate new bone formation when fibrous

25     tissue healing normally is expected. On the other hand, studies with the 2 mm gap allow a determination of whether transgene expression can speed up natural primary bone healing. Controls were also performed in which animals received no DNA (FIG. 9A and FIG. 9B).

30

11.    Gene Transfer Promotes Bone Repair In Vivo

The present inventors surprisingly found that gene transfer into bone progenitor cells in vivo (i.e., cells

35     in the regenerating tissue in the osteotomy gap) could be readily achieved. Currently, the preferred methods for achieving gene transfer generally involve using a fibrous

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collagen implant material soaked in a solution of DNA
shortly before being placed in the site in which one
desires to promote bone growth. ·As the studies presented
herein show, the implant material facilitates the uptake
5    of exogenous plasmid constructs by cells (in the
osteotomy gap) which clearly participate in bone
regeneration/repair.  The transgenes, following cellular
uptake, direct the expression of recombinant
polypeptides, as evidenced by the *in vivo* expression of
10   functional marker gene products.


Further studies are presented herein demonstrating
that the transfer of an osteotropic gene results in
cellular expression of a recombinant osteotropic
15   molecule, which expression is directly associated with
stimulation of new bone formation.  After considering a
relatively large number of candidate genes, a gene
transfer vector coding for a fragment of human
parathyroid hormone (hPTH1-34) was chosen for the
20   inventors' initial studies.  Several factors were
considered in making this choice:  (a), recombinant
hPTH1-34 peptides can be discriminated from any
endogenous rat hormone present in osteotomy tissues;
(b), hPTH1-34 peptides will stimulate new bone formation
25   in Sprague-Dawley rats, indicating that the human peptide
can efficiently bind the PTH/PTHrP receptor on the rat
osteoblast cell surface; and (c), there is only one
PTH/PTHrP receptor, the gene for this receptor has been
cloned, and cDNA probes to the receptor are available.
30

Thus, in terms of understanding the mechanism of
action of the transgene on new bone formation *in vivo*,
the inventors reasoned it most straightforward to
correlate the expression of recombinant hPTH1-34 peptide
35   and its receptor with new bone formation in the rat
osteotomy model.  Of course, following these initial
studies, it is contemplated that any one of a wide

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variety of genes may be employed in connection with the
bone gene transfer embodiments of the present invention.

Previous studies have indicated that hPTH1-34 is a
5    more powerful anabolic agent when given intermittently as
opposed to continuously. Despite the fact that an
anabolic effect would still be expected with continuous
dosing, as documented by the studies of Parsons and co-
workers (Tam et al., 1982; Spencer et al., 1989), there
10   was a concern that the PLJ-hPTH1-34 transgene may not
function very effectively as transfected cells would be
expected to express recombinant hPTH1-34 molecules in a
constitutive manner. The finding that transfection and
expression of the LPH-hPTH1-34 transgene did effectively
15   stimulate bone formation in the rat osteotomy model was
therefore an important result.

As the osteotomy site in this model is highly
vascularized, one possible complication of the studies
20   with the PLJ-hPTH1-34 transgene is the secretion of
recombinant human PTH from the osteotomy site with
consequent hypercalcemia and (potentially) animal death.
Weekly serum calcium levels should therefore be
determined when using this transgene. The fact that no
25   evidence of disturbed serum calcium levels has been found
in this work is therefore a further encouraging finding.

These studies complement others by the inventors in
which direct gene transfer was employed to introduce
30   genes into Achilles' tendon and cruciate ligament, as
described in Example XI.

Various immediate applications for using nucleic
acid delivery in connection with bone disorders became
35   apparent to the inventors following these surprising
findings. The direct transfer of an osteotropic gene to
promote fracture repair in clinical orthopaedic practice

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is just one use.  Other important aspects of this
technology include the use of gene transfer to treat
patients with "weak bones", such as in diseases like
osteoporosis; to improve poor healing which may arise for
5    unknown reasons, *e.g.*, fibrous non-union; to promote
implant integration and the function of artificial
joints; to stimulate healing of other skeletal tissues
such as Achilles' tendon; and as an adjuvant to repair
large defects.  In all such embodiments, DNA is being
10   used as a direct pharmaceutical agent.

## 12. Biological Functional Equivalents

As mentioned above, modification and changes may be
15   made in the structure of an osteotropic gene and still
obtain a functional molecule that encodes a protein or
polypeptide with desirable characteristics.  The
following is a discussion based upon changing the amino
acids of a protein to create an equivalent, or even an
20   improved, second-generation molecule.  The amino acid
changes may be achieved by changing the codons of the DNA
sequence, according to the following codon table:

Table 1

| Amino Acids | | | Codons | | | | | |
|---|---|---|---|---|---|---|---|---|
| Alanine | Ala | A | GCA | GCC | GCG | GCU | | |
| Cysteine | Cys | C | UGC | UGU | | | | |
| Aspartic acid | Asp | D | GAC | GAU | | | | |
| Glutamic acid | Glu | E | GAA | GAG | | | | |
| Phenylalanine | Phe | F | UUC | UUU | | | | |
| Glycine | Gly | G | GGA | GGC | GGG | GGU | | |
| Histidine | His | H | CAC | CAU | | | | |
| Isoleucine | Ile | I | AUA | AUC | AUU | | | |
| Lysine | Lys | K | AAA | AAG | | | | |
| Leucine | Leu | L | UUA | UUG | CUA | CUC | CUG | CUU |
| Methionine | Met | M | AUG | | | | | |
| Asparagine | Asn | N | AAC | AAU | | | | |
| Proline | Pro | P | CCA | CCC | CCG | CCU | | |
| Glutamine | Gln | Q | CAA | CAG | | | | |
| Arginine | Arg | R | AGA | AGG | CGA | CGC | CGG | CGU |
| Serine | Ser | S | AGC | AGU | UCA | UCC | UCG | UCU |
| Threonine | Thr | T | ACA | ACC | ACG | ACU | | |
| Valine | Val | V | GUA | GUC | GUG | GUU | | |
| Tryptophan | Trp | W | UGG | | | | | |
| Tyrosine | Tyr | Y | UAC | UAU | | | | |

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of osteotropic genes without appreciable loss of their biological utility or activity.

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In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in
5    the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for
10   example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge
15   characteristics (Kyte and Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3);
20   proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may
25   be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose
30   hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the
35   substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the

greatest local average hydrophilicity of a protein, as
governed by the hydrophilicity of its adjacent amino
acids, correlates with a biological property of the
protein.

5

As detailed in U.S. Patent 4,554,101, the following
hydrophilicity values have been assigned to amino acid
residues: arginine (+3.0); lysine (+3.0); aspartate
(+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3);
10    asparagine (+0.2); glutamine (+0.2); glycine (0);
threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5);
histidine *-0.5); cysteine (-1.0); methionine (-1.3);
valine (-1.5); leucine (-1.8); isoleucine (-1.8);
tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).
15

It is understood that an amino acid can be
substituted for another having a similar hydrophilicity
value and still obtain a biologically equivalent, and in
particular, an immunologically equivalent protein. In
20    such changes, the substitution of amino acids whose
hydrophilicity values are within ±2 is preferred, those
which are within ±1 are particularly preferred, and those
within ±0.5 are even more particularly preferred.

25    As outlined above, amino acid substitutions are
generally therefore based on the relative similarity of
the amino acid side-chain substituents, for example,
their hydrophobicity, hydrophilicity, charge, size, and
the like. Exemplary substitutions which take various of
30    the foregoing characteristics into consideration are well
known to those of skill in the art and include: arginine
and lysine; glutamate and aspartate; serine and
threonine; glutamine and asparagine; and valine, leucine
and isoleucine.

35

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### 13.  Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in
the preparation of individual peptides, or biologically
5     functional equivalent proteins or peptides, through
specific mutagenesis of the underlying DNA.  The
technique further provides a ready ability to prepare and
test sequence variants, for example, incorporating one or
more of the foregoing considerations, by introducing one
10    or more nucleotide sequence changes into the DNA.  Site-
specific mutagenesis allows the production of mutants
through the use of specific oligonucleotide sequences
which encode the DNA sequence of the desired mutation, as
well as a sufficient number of adjacent nucleotides, to
15    provide a primer sequence of sufficient size and sequence
complexity to form a stable duplex on both sides of the
deletion junction being traversed.  Typically, a primer
of about 17 to 25 nucleotides in length is preferred,
with about 5 to 10 residues on both sides of the junction
20    of the sequence being altered.

In general, the technique of site-specific
mutagenesis is well known in the art, as exemplified by
various publications.  As will be appreciated, the
25    technique typically employs a phage vector which exists
in both a single stranded and double stranded form.
Typical vectors useful in site-directed mutagenesis
include vectors such as the M13 phage.  These phage are
readily commercially available and their use is generally
30    well known to those skilled in the art.  Double stranded
plasmids are also routinely employed in site directed
mutagenesis which eliminates the step of transferring the
gene of interest from a plasmid to a phage.

35    In general, site-directed mutagenesis in accordance
herewith is performed by first obtaining a single-
stranded vector or melting apart of two strands of a

double stranded vector which includes within its sequence
a DNA sequence which encodes the desired osteotropic
protein. An oligonucleotide primer bearing the desired
mutated sequence is prepared, generally synthetically.

5   This primer is then annealed with the single-stranded
vector, and subjected to DNA polymerizing enzymes such as
_E. coli_ polymerase I Klenow fragment, in order to
complete the synthesis of the mutation-bearing strand.
Thus, a heteroduplex is formed wherein one strand encodes

10  the original non-mutated sequence and the second strand
bears the desired mutation. This heteroduplex vector is
then used to transform appropriate cells, such as _E. coli_
cells, and clones are selected which include recombinant
vectors bearing the mutated sequence arrangement.

15

The preparation of sequence variants of the selected
osteotropic gene using site-directed mutagenesis is
provided as a means of producing potentially useful
species and is not meant to be limiting as there are

20  other ways in which sequence variants of osteotropic
genes may be obtained. For example, recombinant vectors
encoding the desired osteotropic gene may be treated with
mutagenic agents, such as hydroxylamine, to obtain
sequence variants.

25

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14.   Monoclonal Antibody Generation

Means for preparing and characterizing antibodies
are well known in the art (See, *e.g.*, Antibodies:  A
5   Laboratory Manual, Cold Spring Harbor Laboratory, 1988;
incorporated herein by reference).

The methods for generating monoclonal antibodies
(MAbs) generally begin along the same lines as those for
10   preparing polyclonal antibodies.  Briefly, a polyclonal
antibody is prepared by immunizing an animal with an
immunogenic composition in accordance with the present
invention and collecting antisera from that immunized
animal.  A wide range of animal species can be used for
15   the production of antisera.  Typically the animal used
for production of anti-antisera is a rabbit, a mouse, a
rat, a hamster, a guinea pig or a goat.  Because of the
relatively large blood volume of rabbits, a rabbit is a
preferred choice for production of polyclonal antibodies.
20

As is well known in the art, a given composition may
vary in its immunogenicity.  It is often necessary
therefore to boost the host immune system, as may be
achieved by coupling a peptide or polypeptide immunogen
25   to a carrier.  Exemplary and preferred carriers are
keyhole limpet hemocyanin (KLH) and bovine serum albumin
(BSA).  Other albumins such as ovalbumin, mouse serum
albumin or rabbit serum albumin can also be used as
carriers.  Means for conjugating a polypeptide to a
30   carrier protein are well known in the art and include
glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide
ester, carbodiimyde and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity
35   of a particular immunogen composition can be enhanced by
the use of non-specific stimulators of the immune
response, known as adjuvants.  Exemplary and preferred

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adjuvants include complete Freund's adjuvant (a non-
specific stimulator of the immune response containing
killed *Mycobacterium tuberculosis*), incomplete Freund's
adjuvants and aluminum hydroxide adjuvant.

5

 The amount of immunogen composition used in the
production of polyclonal antibodies varies upon the
nature of the immunogen as well as the animal used for
immunization.  A variety of routes can be used to

10 administer the immunogen (subcutaneous, intramuscular,
intradermal, intravenous and intraperitoneal).  The
production of polyclonal antibodies may be monitored by
sampling blood of the immunized animal at various points
following immunization.  A second, booster injection, may

15 also be given.  The process of boosting and titering is
repeated until a suitable titer is achieved.  When a
desired level of immunogenicity is obtained, the
immunized animal can be bled and the serum isolated and
stored, and/or the animal can be used to generate MAbs.

20.

 MAbs may be readily prepared through use of well-
known techniques, such as those exemplified in U.S.
Patent 4,196,265, incorporated herein by reference.
Typically, this technique involves immunizing a suitable

25 animal with a selected immunogen composition, e.g., a
purified or partially purified LTBP-3 protein,
polypeptide or peptide.  The immunizing composition is
administered in a manner effective to stimulate antibody
producing cells.  Rodents such as mice and rats are

30 preferred animals, however, the use of rabbit, sheep frog
cells is also possible.  The use of rats may provide
certain advantages (Goding, 1986, pp. 60-61), but mice
are preferred, with the BALB/c mouse being most preferred

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as this is most routinely used and generally gives a
higher percentage of stable fusions.

    Following immunization, somatic cells with the
5    potential for producing antibodies, specifically B
lymphocytes (B cells), are selected for use in the MAb
generating protocol.  These cells may be obtained from
biopsied spleens, tonsils or lymph nodes, or from a
peripheral blood sample.  Spleen cells and peripheral
10   blood cells are preferred, the former because they are a
rich source of antibody-producing cells that are in the
dividing plasmablast stage, and the latter because
peripheral blood is easily accessible.  Often, a panel of
animals will have been immunized and the spleen of animal
15   with the highest antibody titer will be removed and the
spleen lymphocytes obtained by homogenizing the spleen
with a syringe.  Typically, a spleen from an immunized
mouse contains approximately 5 X $10^7$ to 2 X $10^8$
lymphocytes.
20

    The antibody-producing B lymphocytes from the
immunized animal are then fused with cells of an immortal
myeloma cell, generally one of the same species as the
animal that was immunized.  Myeloma cell lines suited for
25   use in hybridoma-producing fusion procedures preferably
are non-antibody-producing, have high fusion efficiency,
and enzyme deficiencies that render then incapable of
growing in certain selective media which support the
growth of only the desired fused cells (hybridomas).
30

    Any one of a number of myeloma cells may be used, as
are known to those of skill in the art (Goding, pp.
65-66, 1986; Campbell, pp. 75-83, 1984).  For example,
where the immunized animal is a mouse, one may use
35   P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO,
NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for
rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and

4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6
are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1
myeloma cell line (also termed P3-NS-1-Ag4-1), which is
readily available from the NIGMS Human Genetic Mutant
Cell Repository by requesting cell line repository number
GM3573. Another mouse myeloma cell line that may be used
is the 8-azaguanine-resistant mouse murine myeloma SP2/0
non-producer cell line.

Methods for generating hybrids of antibody-producing
spleen or lymph node cells and myeloma cells usually
comprise mixing somatic cells with myeloma cells in a 2:1
proportion, though the proportion may vary from about
20:1 to about 1:1, respectively, in the presence of an
agent or agents (chemical or electrical) that promote the
fusion of cell membranes. Fusion methods using Sendai
virus have been described by Kohler and Milstein (1975;
1976), and those using polyethylene glycol (PEG), such as
37% (v/v) PEG, by Gefter et al. (1977). The use of
electrically induced fusion methods is also appropriate
(Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at
low frequencies, about $1 \times 10^{-6}$ to $1 \times 10^{-8}$. However, this
does not pose a problem, as the viable, fused hybrids are
differentiated from the parental, unfused cells
(particularly the unfused myeloma cells that would
normally continue to divide indefinitely) by culturing in
a selective medium. The selective medium is generally
one that contains an agent that blocks the *de novo*
synthesis of nucleotides in the tissue culture media.
Exemplary and preferred agents are aminopterin,
methotrexate, and azaserine. Aminopterin and
methotrexate block *de novo* synthesis of both purines and
pyrimidines, whereas azaserine blocks only purine

synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal

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antibody produced by the fused cell hybrid.  The body
fluids of the animal, such as serum or ascites fluid, can
then be tapped to provide MAbs in high concentration.
The individual cell lines could also be cultured *in*

5    *vitro*, where the MAbs are naturally secreted into the
culture medium from which they can be readily obtained in
high concentrations.  MAbs produced by either means may
be further purified, if desired, using filtration,
centrifugation and various chromatographic methods such

10   as HPLC or affinity chromatography.


**15.  LTBP-3**


Other aspects of the present invention concern

15   isolated DNA segments and recombinant vectors encoding
LTBP-3, and the creation and use of recombinant host
cells through the application of DNA technology, that
express LTBP-3 gene products.  As such, the invention
concerns DNA segment comprising an isolated gene that

20   encodes a protein or peptide that includes an amino acid
sequence essentially as set forth by a contiguous
sequence from SEQ ID NO:3.  These DNA segments are
represented by those that include a nucleic acid sequence
essentially as set forth by a contiguous sequence from

25   SEQ ID NO:2 (FIG. 25).  Compositions that include a
purified protein that has an amino acid sequence
essentially as set forth by the amino acid sequence of
SEQ ID NO:3 (FIG. 26) are also encompassed by the
invention.

30

The TGF-βs represent a family of structurally
related molecules with diverse effects on mammalian cell
shape, growth, and differentiation (Roberts and Sporn,
1990).  Initially synthesized as a precursor consisting

35   of an amino-terminal propeptide followed by mature TGF-β,
two chains of nascent pro-TGF-β associate in most tissues
to form a Mr ~106,000 inactive disulfide-bonded dimer.

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Homodimers are most common, but heterodimers have also
been described (Cheifetz et al., 1987; Ogawa et al.,
1992). During biosynthesis the mature TGF-β dimer is
cleaved from the propeptide dimer. TGF-β latency results
5    in part from the non-covalent association of propeptide
and mature TGF-β dimers (Pircher et al., 1984, 1986;
Wakefield et al., 1987; Millan et al., 1992; Miyazono and
Heldin, 1989). Consequently, the propeptide dimer is
often referred to as the latency associated protein
10   (LAP), and LAP plus the disulfide-bonded TGF-β dimer are
also known as the small latent complex. In the
extracellular space small latent complexes must be
dissociated to activate mature TGF-β. The mechanism of
activation of the latent complex is thought to be one of
15   the most important steps governing TGF-β effects (Lyons
et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et
al., 1990; Sato et al., 1993).


In certain lines of cultured cells small latent
20   growth factor complexes may contain additional high
molecular weight proteins. The best characterized of
these high molecular weight proteins is the latent TGF-β
binding protein, or LTBP (Miyazono et al., 1988; Kanzaki
et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992;
25   Taketazu et al., 1994). LTBP produced by different cell
types is heterogeneous in size, perhaps because of
alternative splicing or because of tissue-specific
proteolytic processing (Miyazono et al., 1988; Wakefield
et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990).
30   Latent TGF-β complexes that contain LTBP are known as
large latent complexes. LTBP has no known covalent
linkage to mature   TGF-β, but rather it is linked by a
disulfide bond to LAP.

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Regarding the novel protein LTBP-3, the present
invention concerns DNA segments, that can be isolated
from virtually any mammalian source, that are free from
5      total genomic DNA and that encode proteins having LTBP-3-
like activity.  DNA segments encoding LTBP-3-like species
may prove to encode proteins, polypeptides, subunits,
functional domains, and the like.

10     As used herein, the term "DNA segment" refers to a
DNA molecule that has been isolated free of total genomic
DNA of a particular species.  Therefore, a DNA segment
encoding LTBP-3 refers to a DNA segment that contains
LTBP-3 coding sequences yet is isolated away from, or
15     purified free from, total genomic DNA of the species from
which the DNA segment is obtained.  Included within the
term "DNA segment", are DNA segments and smaller
fragments of such segments, and also recombinant vectors,
including, for example, plasmids, cosmids, phagemids,
20     phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or
purified LTBP-3 gene refers to a DNA segment including
LTBP-3 coding sequences and, in certain aspects,
25     regulatory sequences, isolated substantially away from
other naturally occurring genes or protein encoding
sequences.  In this respect, the term "gene" is used for
simplicity to refer to a functional protein, polypeptide
or peptide encoding unit.  As will be understood by those
30     in the art, this functional term includes both genomic
sequences, cDNA sequences and smaller engineered gene
segments that express, or may be adapted to express,
proteins, polypeptides or peptides.

35     "Isolated substantially away from other coding
sequences" means that the gene of interest, in this case,
a gene encoding LTBP-3, forms the significant part of the

coding region of the DNA segment, and that the DNA
segment does not contain large portions of naturally-
occurring coding DNA, such as large chromosomal fragments
or other functional genes or cDNA coding regions.  Of

5     course, this refers to the DNA segment as originally
isolated, and does not exclude genes or coding regions
later added to the segment by the hand of man.  :

In particular embodiments, the invention concerns

10    isolated DNA segments and recombinant vectors
incorporating DNA sequences that encode an LTBP-3 species
that includes within its amino acid sequence an amino
acid sequence essentially as set forth in SEQ ID NO:3.
In other particular embodiments, the invention concerns

15    isolated DNA segments and recombinant vectors
incorporating DNA sequences that include within their
sequence a nucleotide sequence essentially as set forth
in SEQ ID NO:2.

20         The term "a sequence essentially as set forth in SEQ
ID NO:3" means that the sequence substantially
corresponds to a portion of SEQ ID NO:3 and has
relatively few amino acids that are not identical to, or
a biologically functional equivalent of, the amino acids

25    of SEQ ID NO:3.  The term "biologically functional
equivalent" is well understood in the art and is further
defined in detail herein (for example, see section 7,
preferred embodiments).  Accordingly, sequences that have
between about 70% and about 80%; or more preferably,

30    between about 81% and about 90%; or even more preferably,
between about 91% and about 99%; of amino acids that are
identical or functionally equivalent to the amino acids
of SEQ ID NO:3 will be sequences that are "essentially as
set forth in SEQ ID NO:3".

35
In certain other embodiments, the invention concerns
isolated DNA segments and recombinant vectors that

include within their sequence a nucleic acid sequence
essentially as set forth in SEQ ID NO:2.  The term
"essentially as set forth in SEQ ID NO:2" is used in the
same sense as described above and means that the nucleic

5       acid sequence substantially corresponds to a portion of
SEQ ID NO:2 and has relatively few codons that are not
identical, or functionally equivalent, to the codons of
SEQ ID NO:2.  Again, DNA segments that encode proteins
exhibiting LTBP-3-like activity will be most preferred.

10

It will also be understood that amino acid and
nucleic acid sequences may include additional residues,
such as additional N- or C-terminal amino acids or 5' or
3' sequences, and yet still be essentially as set forth

15      in one of the sequences disclosed herein, so long as the
sequence meets the criteria set forth above, including
the maintenance of biological protein activity where
protein expression is concerned.  The addition of
terminal sequences particularly applies to nucleic acid

20      sequences that may, for example, include various non-
coding sequences flanking either of the 5' or 3' portions
of the coding region or may include various internal
sequences, i.e., introns, which are known to occur within
genes.

25

Naturally, the present invention also encompasses
DNA segments that are complementary, or essentially
complementary, to the sequence set forth in SEQ ID NO:2.
Nucleic acid sequences that are "complementary" are those

30      that are capable of base-pairing according to the
standard Watson-Crick complementarity rules.  As used
herein, the term "complementary sequences" means nucleic
acid sequences that are substantially complementary, as
may be assessed by the same nucleotide comparison set

35      forth above, or as defined as being capable of
hybridizing to the nucleic acid segment of SEQ ID NO:2,

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under relatively stringent conditions such as those
described herein.

The nucleic acid segments of the present invention,
5    regardless of the length of the coding sequence itself,
may be combined with other DNA sequences, such as
promoters, polyadenylation signals, additional
restriction enzyme sites, multiple cloning sites, other
coding segments, and the like, such that their overall
10   length may vary considerably. It is therefore
contemplated that a nucleic acid fragment of almost any
length may be employed, with the total length preferably
being limited by the ease of preparation and use in the
intended recombinant DNA protocol. For example, nucleic
15   acid fragments may be prepared that include a short
contiguous stretch identical to or complementary to SEQ
ID NO:2, such as about 14 nucleotides, and that are up to
about 10,000 or about 5,000 base pairs in length, with
segments of about 3,000 being preferred in certain cases.
20   DNA segments with total lengths of about 1,000, about
500, about 200, about 100 and about 50 base pairs in
length (including all intermediate lengths) are also
contemplated to be useful.

25   It will be readily understood that "intermediate
lengths", in these contexts, means any length between the
quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.;
21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.;
100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.;
30   including all integers through the 200-500; 500-1,000;
1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000
ranges, up to and including sequences of about 12,001,
12,002, 13,001, 13,002 and the like.

35   It will also be understood that this invention is
not limited to the particular nucleic acid and amino acid
sequences of SEQ ID NO:2 and SEQ ID NO:3. Recombinant

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vectors and isolated DNA segments may therefore variously
include the LTBP-3 coding regions themselves, coding
regions bearing selected alterations or modifications in
the basic coding region, or they may encode larger
5   polypeptides that nevertheless include LTBP-3-coding
regions or may encode biologically functional equivalent
proteins or peptides that have variant amino acids
sequences.

10      The DNA segments of the present invention encompass
biologically functional equivalent LTBP-3 proteins and
peptides.  Such sequences may arise as a consequence of
codon redundancy and functional equivalency that are
known to occur naturally within nucleic acid sequences
15  and the proteins thus encoded.  Alternatively,
functionally equivalent proteins or peptides may be
created via the application of recombinant DNA
technology, in which changes in the protein structure may
be engineered, based on considerations of the properties
20  of the amino acids being exchanged.  Changes designed by
man may be introduced through the application of site-
directed mutagenesis techniques, e.g., to introduce
improvements to the antigenicity of the protein or to
test mutants in order to examine activity at the
25  molecular level.

If desired, one may also prepare fusion proteins and
peptides, e.g., where the LTBP-3 coding regions are
aligned within the same expression unit with other
30  proteins or peptides having desired functions, such as
for purification or immunodetection purposes (e.g.,
proteins that may be purified by affinity chromatography
and enzyme label coding regions, respectively).

35      Recombinant vectors form further aspects of the
present invention.  Particularly useful vectors are
contemplated to be those vectors in which the coding

portion of the DNA segment, whether encoding a full
length protein or smaller peptide, is positioned under
the control of a promoter.  The promoter may be in the
form of the promoter that is naturally associated with a
5    LTBP-3 gene, as may be obtained by isolating the 5' non-
coding sequences located upstream of the coding segment
or exon, for example, using recombinant cloning and/or
PCR™ technology, in connection with the compositions
disclosed herein.
10

     In other embodiments, it is contemplated that
certain advantages will be gained by positioning the
coding DNA segment under the control of a recombinant, or
heterologous, promoter.  As used herein, a recombinant or
15   heterologous promoter is intended to refer to a promoter
that is not normally associated with an LTBP-3 gene in
its natural environment.  Such promoters may include
LTBP-3 promoters normally associated with other genes,
and/or promoters isolated from any bacterial, viral,
20   eukaryotic, or mammalian cell.  Naturally, it will be
important to employ a promoter that effectively directs
the expression of the DNA segment in the cell type,
organism, or even animal, chosen for expression.  The use
of promoter and cell type combinations for protein
25   expression is generally known to those of skill in the
art of molecular biology, for example, see Sambrook et
al., 1989.  The promoters employed may be constitutive,
or inducible, and can be used under the appropriate
conditions to direct high level expression of the
30   introduced DNA segment, such as is advantageous in the
large-scale production of recombinant proteins or
peptides.  Appropriate promoter systems contemplated for
use in high-level expression include, but are not limited

to, the *Pichia* expression vector system (Pharmacia LKB
Biotechnology) (see Example XVI herein).

5    In connection with expression embodiments to prepare
recombinant LTBP-3 proteins and peptides, it is
contemplated that longer DNA segments will most often be
used, with DNA segments encoding the entire LTBP-3
protein or functional domains, subunits, *etc.* being most
preferred.  However, it will be appreciated that the use
10   of shorter DNA segments to direct the expression of LTBP-
3 peptides or epitopic core regions, such as may be used
to generate anti-LTBP-3 antibodies, also falls within the
scope of the invention.  DNA segments that encode peptide
antigens from about 15 to about 50 amino acids in length,
15   or more preferably, from about 15 to about 30 amino acids
in length are contemplated to be particularly useful.

The LTBP-3 gene and DNA segments may also be used in
connection with somatic expression in an animal or in the
20   creation of a transgenic animal.  Again, in such
embodiments, the use of a recombinant vector that directs
the expression of the full length or active LTBP-3
protein is particularly contemplated.

25   In addition to their use in directing the expression
of the LTBP-3 protein, the nucleic acid sequences
disclosed herein also have a variety of other uses.  For
example, they also have utility as probes or primers in
nucleic acid hybridization embodiments.  As such, it is
30   contemplated that nucleic acid segments that comprise a
sequence region that consists of at least a 14 nucleotide
long contiguous sequence that has the same sequence as,
or is complementary to, a 14 nucleotide long contiguous
sequence of SEQ ID NO:2 will find particular utility.
35   Longer contiguous identical or complementary sequences,
*e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000

(including all intermediate lengths) and even up to full
length sequences will also be of use in certain
embodiments.

5          The ability of such nucleic acid probes to
specifically hybridize to LTBP-3-encoding sequences will
enable them to be of use in detecting the presence of
complementary sequences in a given sample. However,
other uses are envisioned, including the use of the
10       sequence information for the preparation of mutant
species primers, or primers for use in preparing other
genetic constructions.

          Nucleic acid molecules having sequence regions
15       consisting of contiguous nucleotide stretches of 10-14,
15-20, 30, 50, or even of 100-200 nucleotides or so,
identical or complementary to SEQ ID NO:2, are
particularly contemplated as hybridization probes for use
in, e.g., Southern and Northern blotting. This would
20       allow LTBP-3 structural or regulatory genes to be
analyzed, both in diverse cell types and also in various
mammalian cells. The total size of fragment, as well as
the size of the complementary stretch(es), will
ultimately depend on the intended use or application of
25       the particular nucleic acid segment. Smaller fragments
will generally find use in hybridization embodiments,
wherein the length of the contiguous complementary region
may be varied, such as between about 10-14 and about 100
nucleotides, but larger contiguous complementarity
30       stretches may be used, according to the length
complementary sequences one wishes to detect.

          The use of a hybridization probe of about 10-14
nucleotides in length allows the formation of a duplex
35       molecule that is both stable and selective. Molecules
having contiguous complementary sequences over stretches
greater than 10 bases in length are generally preferred,

though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All that is required is to review the sequence set forth in SEQ ID NO:2 and to select any continuous portion of the sequence, from about 10-14 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors, such as, by way of example only, one may wish to employ primers from towards the termini of the total sequence.

The process of selecting and preparing a nucleic acid segment that includes a contiguous sequence from within SEQ ID NO:2 may alternatively be described as preparing a nucleic acid fragment. Of course, fragments may also be obtained by other techniques such as, *e.g.*, by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,603,102 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

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Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of LTBP-3 gene or cDNA fragments. Depending on the

5 application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent

10 conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of 50°C to 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and

15 the template or target strand, and would be particularly suitable for isolating LTBP-3 genes.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer

20 strand hybridized to an underlying template or where one seeks to isolate LTBP-3-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex.

25 In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control

30 hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions

35 can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

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In certain embodiments, it will be advantageous to
employ nucleic acid sequences of the present invention in
combination with an appropriate means, such as a label,
for determining hybridization. A wide variety of
5     appropriate indicator means are known in the art,
including fluorescent, radioactive, enzymatic or other
ligands, such as avidin/biotin, which are capable of
giving a detectable signal. In preferred embodiments,
one will likely desire to employ a fluorescent label or
10    an enzyme tag, such as urease, alkaline phosphatase or
peroxidase, instead of radioactive or other environmental
undesirable reagents. In the case of enzyme tags,
colorimetric indicator substrates are known that can be
employed to provide a means visible to the human eye or
15    spectrophotometrically, to identify specific
hybridization with complementary nucleic acid-containing
samples.

In general, it is envisioned that the hybridization
20    probes described herein will be useful both as reagents
in solution hybridization as well as in embodiments
employing a solid phase. In embodiments involving a
solid phase, the test DNA (or RNA) is adsorbed or
otherwise affixed to a selected matrix or surface. This
25    fixed, single-stranded nucleic acid is then subjected to
specific hybridization with selected probes under desired
conditions. The selected conditions will depend on the
particular circumstances based on the particular criteria
required (depending, for example, on the G+C content,
30    type of target nucleic acid, source of nucleic acid, size
of hybridization probe, etc.). Following washing of the
hybridized surface so as to remove nonspecifically bound
probe molecules, specific hybridization is detected, or
even quantitated, by means of the label.
35

The following examples are included to demonstrate
preferred embodiments of the invention. It should be

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appreciated by those of skill in the art that the
techniques disclosed in the examples which follow
represent techniques discovered by the inventor to
function well in the practice of the invention, and thus
5   can be considered to constitute preferred modes for its
practice.  However, those of skill in the art should, in
light of the present disclosure, appreciate that many
changes can be made in the specific embodiments which are
disclosed and still obtain a like or similar result
10  without departing from the spirit and scope of the
invention.


## EXAMPLE I
### ANIMAL MODEL FOR ASSESSING NEW BONE FORMATION

15

As various animal models were not suitable for
studying the effects of nucleic acid transfer on bone
formation, the inventors employed the following model
system.  The important features of the rat osteotomy
20  model are as described in the following protocol (which
is generally completed in 25-35 minutes).


The osteotomy was performed on one femur per animal.
Right to left differences have not been apparent, but
25  such differences are monitored in these studies, since
the limb receiving the osteotomy is randomized.


After pre-operative preparation (i.e., shaving and
Betadine® scrub), adult male Sprague Dawley rats (~500
30  gm, retired male breeders) were anesthetized using a 3%
halothane 97% oxygen mixture (700 ml/min. flow rate).  A
lateral approach to the femur was made on one limb.
Utilizing specially designed surgical guides, four 1.2-mm
diameter pins were screwed into the diaphysis after pre-
35  drilling with a high speed precision bit.  A surgical
template ensured precise and parallel placement of the
pins.  The order of pin placement was always the same:

outer proximal first and then outer distal, inner proximal and inner distal (with "outer" and "inner" referring to the distance from the hip joint). Pin placement in the center of the femur was ensured by

5    fluoroscopic imaging during pin placement. The external fixator was secured on the pins and a t mm or 2 mm segmental defect was created in the central diaphysis through an incision using a Hall Micro 100 Oscillating saw (#5053-60 Hall surgical blades) under constant

10   irrigation. Other than the size of the segmental defect, there is no difference between the 5 mm and 2 mm osteotomy protocols (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

15

The contents of the osteotomy site were irrigated with sterile saline and the fibrous collagen implant material, previously soaked in a solution of plasmid DNA or other DNA construct, if appropriate, was placed in

20   situ. The wound was then closed in layers. Since the fixator provided the necessary stability no limitations on animal ambulation existed, and other supports were not required. The surgical protocol has been successfully performed on 53 animals to date, including 35 controls

25   (Table 2 and FIG. 24). None of these animals have died and no significant adverse effects have been observed, other than complications that might be associated with surgical fracture repair. Minor complications that were experienced include 1 animal that developed a post-

30   operative osteomyelitis and 1 animal in which 2/4 pins loosened as a consequence of post-operative bone fracture.

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## EXAMPLE II

### IMPLANT MATERIAL FOR USE IN BONE GENE TRANSFER

Various implant materials may be used for
5   transferring genes into the site of bone repair and/or
regeneration *in vivo*. These materials are soaked in a
solution containing the DNA or gene that is to be
transferred to the bone regrowth site. Alternatively,
DNA may be incorporated into the matrix as a preferred
10  method of making.

One particular example of a suitable material is
fibrous collagen, which may be lyophilized following
extraction and partial purification from tissue and then
15  sterilized. A particularly preferred collagen is the
fibrous collagen implant material termed UltraFiber™, as
may be obtained from Norian Corp., (Mountain View, CA).
Detailed descriptions of the composition and use of
UltraFiber™ are provided in Gunasekaran *et al*., (1993a,
20  b; each incorporated herein by reference).

A more particularly preferred collagen is type II
collagen, with most particularly preferred collagen being
either recombinant type II collagen, or mineralized type
25  II collagen. Prior to placement in osteotomy sites,
implant materials are soaked in solutions of DNA (or
virus) under sterile conditions. The soaking may be for
any appropriate and convenient period, *e.g.*, from 6
minutes to over-night. The DNA (*e.g.*, plasmid) solution
30  will be a sterile aqueous solution, such as sterile water
or an acceptable buffer, with the concentration generally
being about 0.5 - 1.0 mg/ml. Currently preferred
plasmids are those such as pGL2 (Promega), pSV40$\beta$-gal,
pAd.CMV*lacZ*, and pLJ.

35

## EXAMPLE III
## PARATHYROID HORMONE GENE CONSTRUCTS

5       The active fragment of the human parathyroid hormone
gene (hPTH1-34) was chosen as the first of the
osteotropic genes to be incorporated into an expression
vector for use in gene transfer to promote new bone
formation in the rat osteotomy model.

10      The inventors chose to construct the hPTH1-34
transgene in the pLJ expression vector (FIG. 10), since
this vector was appropriate for studies of transgene
function both *in vitro* and *in vivo*. A schematic of the
PLJ-hPTH1-34 transgene is shown in FIG. 10. The DNA and
15      amino acid sequences of the hPTH1-34 are well known,
*e.g.*, see Hendy *et al.*, (1981, incorporated herein by
reference). To insert the transgene into the PLJ
expression vector PCR™ of a full-length PTH recombinant
clone was employed, followed by standard molecular
20      biological manipulation.

        A retroviral stock was then generated following
CaPO$_4$-mediated transfection of $\phi$ crip cells with the PLJ-
hPTH1-34 construct, all according to standard protocols
25      (Sambrook *et al.*, 1989). Independent transduced Rat-1
clones were obtained by standard infection and selection
procedures (Sambrook *et al.*, 1989).

        One clone (YZ-15) was analyzed by Southern analysis,
30      demonstrating that the PLJ-hPTH1-34 transgene had stably
integrated into the Rat-1 genome (FIG. 11). A Northern
analysis was next performed to show that the YZ-15 clone
expressed the PLJ-hPTH1-34 transgene, as evidenced by the
presence of specific PLJ-hPTH1-34 transcripts (FIG. 12).

35

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EXAMPLE IV

PARATHYROID HORMONE POLYPEPTIDE EXPRESSION AND ACTIVITY

A sensitive and specific radioimmunoassay was
5   performed to demonstrate that the YZ-15 cells expressed
and secreted a recombinant hPTH1-34 molecule (Table 2).
The radioimmunoassay was performed on media from
transduced Rat-1 clones.  To quantify secretion of the
recombinant hPTH-1-34 peptide produced by YZ-15 cells,
10  the culture medium from one 100 mm confluent dish was
collected over a 24 hour period and assayed with the NH2-
terminal hPTH RIA kit (Nichols Institute Diagnostics)
according to the manufacturer's protocol.  PLJ-hPTH1-87
cells and BAG cells served as positive and negative
15  controls, respectively.

Protein concentrations in Table 2 are expressed as
the average of three assays plus the standard deviation
(in parenthesis).  The concentration of the 1-34 and full
20  length (1-84) peptides was determined relative to a
standard curve generated with commercially available
reagents (Nichols Institute Diagnostics).

### Table 2

| CELL LINES | PTH  (pg/ml) |
|------------|--------------|
| YZ-15 | 247  (± 38) |
| PLJ-hPTH1-84 | 2616 (± 372) |
| BAG | 13  (± 3) |

30

As shown in Table 2, PTH expression was detected in
both YZ-15 cells and PLJ-hPTH1-84 cells.  BAG cells
produced no detectable PTH and served as a baseline for
the RIA.  These results demonstrate that YZ-15 cells
35  expressed recombinant hPTH1-34 protein.

The recombinant hPTH1-34 molecule was added to rat

osteosarcoma cells and a cAMP response assay conducted in
order to determine whether the secreted molecule had
biological activity.  Unconcentrated media was collected
from YZ-15 cells, PLJ-hPTH1-84 cells, and BAG cells and

5      was used to treat ROS17/2.8 cells for 10 minutes, as
described (Majmudar et al., 1991).  cAMP was then
extracted from treated cells and quantified by RIA (Table
3).  The amount of cAMP shown is the average of three
assays.  The standard deviation of the mean is shown in

10     parenthesis.


### Table 3

| CELL LINES | cAMP (pmol) |
|---|---|
| YZ-15 | 20.3 (± 0.25) |
| PLJ-hPTH184 | 88.5 (± 4.50) |
| BAG | 7.6 (± 0.30) |


A cAMP response was induced by the recombinant PTH

20     secreted by the YZ-15 cells and by PLJ-hPTH1-84 cells.
BAG cells produced no PTH and served as the baseline for
the cAMP assay.  These results provide direct in vitro
evidence that the PLJ-hPTH1-34 transgene directs the
expression and secretion of a functional osteotropic

25     agent.


### EXAMPLE V
### BONE MORPHOGENETIC PROTEIN (BMP) GENE CONSTRUCTS


30     The murine bone morphogenetic protein-4 (BMP-4) was
chosen as the next of the osteotropic genes to be
incorporated into an expression vector for use in
promoting bone repair and regeneration.


35     A full length murine BMP-4 cDNA was generated by
screening a murine 3T3 cell cDNA library (Stratagene).
The human sequence for BMP-4 is well known to those of

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skill in the art and has been deposited in Genbank.
Degenerate oligonucleotide primers were prepared and
employed in a standard PCR™ to obtain a murine cDNA
sequence.

5

The ends of the cDNA clone were further modified
using the polymerase chain reaction so that the full
length cDNA (5'→3' direction) encodes the natural murine
initiator Met codon, the full length murine coding
10    sequence, a 9 amino acid tag (known as the HA epitope),
and the natural murine stop codon.  The amino acid
sequence encoded by the murine BMP-4 transgene is shown
in FIG. 24; this entire sequence, including the tag, is
represented by SEQ ID NO:1.

15

Placement of the HA epitope at the extreme carboxy
terminus should not interfere with the function of the
recombinant molecule sequence *in vitro* or *in vivo*.  The
advantage of the epitope is for utilization in
20    immunohistochemical methods to specifically identify the
recombinant murine BMP-4 molecule in osteotomy tissues *in
vivo*, *e.g.*, the epitope can be identified using a
commercially available monoclonal antibody (Boehringer-
Mannheim), as described herein.

25

Studies to demonstrate that the murine BMP-4
transgene codes for a functional osteotropic agent
include, for example, (a) transfection of COS cells and
immunoprecipitation of a protein band of the correct size
30    using a monoclonal anti-HA antibody (Boehringer-
Mannheim); and (b) a quantitative *in vivo* bone induction
bioassay (Sampath and Reddi, 1981) that involves
implanting proteins from the medium of transfected COS
cells beneath the skin of male rats and scoring for new
35    bone formation in the ectopic site.

EXAMPLE VI

DETECTION OF mRNA BY TISSUE *IN SITU* HYBRIDIZATION

The following technique describes the detection of
5    mRNA in tissue obtained from the site of bone
regeneration.  This may be useful for detecting
expression of the transgene mRNA itself, and also in
detecting expression of hormone or growth factor
receptors or other molecules.  This method may be used in
10   place of, or in addition to, Northern analyses, such as
those described in FIG. 13.


DNA from a plasmid containing the gene for which
mRNA is to be detected is linearized, extracted, and
15   precipitated with ethanol.  Sense and antisense
transcripts are generated from 1 mg template with T3 and
T7 polymerases, *e.g.*, in the presence of [$^{35}$S] UTP at >6
mCi/ml (Amersham Corp., >1200 Ci/mmol) and 1.6 U/ml
RNasin (Promega), with the remaining *in vitro*
20   transcription reagents provided in a kit (SureSite,
Novagen Inc.).  After transcription at 37°C for 1 hour,
DNA templates are removed by a 15 minute digestion at
37°C with 0.5 U/ml RNase-free DNase I, extracted, and
precipitated with ethanol.  Riboprobes are hydrolyzed to
25   an average final length of 150 bp by incubating in 40 mM
NaHCO$_3$, 60 mM Na$_2$CO$_3$, 80 mM DTT at 60°C, according to
previously determined formula.  Hydrolysis is terminated
by addition of sodium acetate,  pH 6.0, and glacial
acetic acid to 0.09 M and 0.005% (v/v), respectively, and
30   the probes are then ethanol precipitated, dissolved in
0.1 M DTT, counted, and stored at -20°C until use.


RNase precautions are taken in all stages of slide
preparation.  Bouins fixed, paraffin embedded tissue
35   sections are heated to 65°C for 10 minutes,
deparaffinized in 3 changes of xylene for 5 minutes, and
rehydrated in a descending ethanol series, ending in

phosphate-buffered saline (PBS). Slides will be soaked
in 0.2 N HCl for 5 min., rinsed in PBS, digested with
0.0002% proteinase K in PBS for 30 minutes at 37°C and
rinsed briefly with DEPC-treated water. After

5   equilibrating for 3 minutes in 0.1 M triethanolamine-HCl
(TEA-HCl), pH 8.0, sections are acetylated in 0.25% (v/v)
acetic anhydride in 0.1 M TEA-HCl for 10 minutes at room
temperature, rinsed in PBS, and dehydrated in an
ascending ethanol series. Each section receives 100-200

10  ml prehybridization solution (0.5 mg/ml denatured RNase-
free tRNA (Boehringer-Mannheim), 10 mM DTT, 5 mg/ml
denatured, sulfurylated salmon sperm DNA, 50% formamide,
10% dextran sulfate, 300 mM NaCl, 1X RNase-free
Denhardt's solution (made with RNase-free bovine serum

15  albumin, Sigma), 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and
then incubate on a 50°C slide warmer in a humidified
enclosure for 2 hours. The sulfurylated salmon-sperm DNA
blocking reagent is used in both prehybridization and
hybridization solutions to help reduce nonspecific

20  binding to tissue by $^{35}$SH groups on the probe. It is
prepared by labeling RNase-free salmon sperm DNA (Sigma)
with non-radioactive α-thio-dCTP and α-thio-dATP
(Amersham) in a standard random oligonucleotide-primed
DNA labeling reaction. Excess prehybridization solution

25  is removed with a brief rinse in 4X SSC before
application of probe.

Riboprobes, fresh tRNA and sulfurylated salmon sperm
DNA will be denatured for 10 minutes at 70°C, and chilled
30  on ice. Hybridization solution, identical to
prehybridization solution except with denatured probe
added to 5 × 10$^6$ CPM/ml, is applied and slides incubated
at 50°C overnight in sealed humidified chambers on a
slide warmer. Sense and antisense probes are applied to
35  serial sections. Slides are rinsed 3 times in 4X SSC,
washed with 2X SSC, 1 mM DTT for 30 min. at 50°C,
digested with RNase A (20 mg/ml RNase A, 0.5 M NaCl, 10

mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 30 min. at 37°C, and rinsed briefly with 2X SSC, 1 mM DTT. Three additional washes are performed, each at 50°C for 30 minutes: once in 2X SSC, 50% formamide, 1 mM DTT, and

5 twice in 1X SSC, 0.13% (w/v) sodium pyrophosphate, 1 mM DTT.

Slides are dehydrated in an ascending ethanol series (with supplementation of the dilute ethanols (50% and

10 70%) with SSC and DTT to 0.1X and 1 mM, respectively). Slides are exposed to X-ray film for 20-60 hours to visualize overall hybridization patterns, dipped in autoradiographic emulsion (Kodak NTB-2, diluted to 50% with 0.3 M ammonium acetate), slowly dried for 2 hours,

15 and exposed (4°C) for periods ranging from 8 days to 8 weeks. After developing emulsion, sections are counter strained with hematoxylin and eosin, dehydrated, and mounted with xylene-based medium. The hybridization signal is visualized under darkfield microscopy.

20

The above *in situ* hybridization protocol may be used, for example, in detecting the temporal and spatial pattern of PTH/PTHrP receptor expression. A suitable rat PTH/PTHrP receptor cDNA probe (R15B) is one that consists

25 of a 1810 bp region encoding the full length rat bone PTH/PTHrP receptor (Abou-Samra *et al.*, 1992). The cDNA fragment is subcloned into pcDNA 1 (Invitrogen Corp., San Diego, CA) and is cut out using *Xba*I and *Bam*HI. This probe has provided positive signals for northern blot

30 analysis of rat, murine, and human osteoblastic cell lines, rat primary calvarial cells, and murine bone tissue. The pcDNA I plasmid contains a T7 and SP6 promoter that facilitate the generation of cRNA probes for *in situ* hybridization. The full length transcript

35 has been used to detect PTH/PTHrP receptor in sections of bone (Lee *et al.*, 1994). The PTHrP cDNA probe (Yasuda *et al.*, 1989) is a 400 bp subcloned fragment in

pBluescript (Stratagene). This probe has been used for *in situ* hybridization, generating an antisense cRNA probe using *Bam*HI cleavage and the T3 primer and a sense cRNA probe using *Eco*RI cleavage and the T7 primer.

## EXAMPLE VII

### *IN VIVO* PROTEIN DETECTION FOLLOWING TRANSGENE EXPRESSION

1.    *β*-galactosidase Transgene

Bacterial *β*-galactosidase can be detected immunohistochemically. Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the bacterial *β*-galactosidase protein.

For immunohistochemistry, cross-Sections (2-3 mm thick) were transferred to poly-L-Lysine coated microscope slides and fixed in acetone at 0°C for at least 20 min. Sections were rehydrated in PBS. Endogenous peroxidase activity was quenched by immersion of tissue sections in 0.1% hydrogen peroxide (in 95% methanol) at room temperature for 10 min, and quenched sections were washed 3x in PBS. In some cases, sectioned calvariae were demineralized by immersion in 4% EDTA, 5% polyvinyl pyrrolidone, and 7% sucrose, pH 7.4, for 24 h at 4°C. Demineralized sections were washed 3x before application for antibodies. Primary antibodies were used without dilution in the form of hybridoma supernatant. Purified antibodies were applied to tissue sections at a concentration of 5 mg/ml. Primary antibodies were detected with biotinylated rabbit antimouse IgG and peroxidase conjugated streptavidin (Zymed Histostain-SPkit). After peroxidase staining, sections were counterstained with hematoxylin.

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Bacterial β-gal can also be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

5

## 2. Luciferase Transgene

Luciferase can be detected by substrate utilization assays. This is conducted using commercially available
10   kits (e.g., Promega) according to the manufacturers' instructions.


## 3. PTH Transgenes
15

Recombinant PTH, such as hPTH1-34 peptide, is assayed in homogenates of osteotomy gap tissue, for example, using two commercially available radioimmunoassay kits according to the manufacturer's
20   protocols (Nichols Institute Diagnostics, San Juan Capistrano, CA).


One kit is the Intact PTH-Parathyroid Hormone 100T Kit. This radioimmunoassay utilizes an antibody to the
25   carboxy terminus of the intact hormone, and thus is used to measure endogenous levels of hormone in gap osteotomy tissue. This assay may be used to establish a baseline value PTH expression in the rat osteotomy model.

30      The second kit is a two-site immunoradiometric kit for the measurement of rat PTH. This kit uses affinity purified antibodies specific for the amino terminus of the intact rat hormone (PTH1-34) and thus will measure endogenous PTH production as well as the recombinant
35   protein. Previous studies have shown that these antibodies cross-react with human PTH and thus are able to recognize recombinant molecules *in vivo*.

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Values obtained with kit #1 (antibody to the carboxy terminus) are subtracted from values obtained with kit #2 (antibody to the amino terminus) to obtain an accurate and sensitive measurements.  The level of recombinant
5   peptide is thus correlated with the degree of new bone formation.


4.   BMP Transgene

10      Preferably, BMP proteins, such as the murine BMP-4 transgene peptide product, are detected immunohistochemically using a specific antibody that recognizes the HA epitope (Majmudar et al., 1991), such as the monoclonal antibody available from Boehringer-
15   Mannheim.  Antibodies to BMP proteins themselves may also be used.  Such antibodies, along with various immunoassay methods, are described in U.S. Patent 4,857,456, incorporated herein by reference.


20      Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane.  One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the recombinant murine BMP-4 molecule.
25


EXAMPLE VIII
DIRECT GENE TRANSFER INTO REGENERATING BONE *IN VIVO*


30      To assess the feasibility of direct gene transfer into regenerating bone *in vivo*, marker gene transfer into cells in the rat osteotomy model was employed.  These studies involved two marker genes: bacterial $\beta$-galactosidase and insect luciferase.
35

Aliquots of a fibrous collagen implant material were soaked in solutions of pure marker gene DNA.  The implant

materials were then placed in the osteotomy site, and
their expression determined as described above.

It was found that both marker genes were
successfully transferred and expressed, without any
failures, as demonstrated by substrate utilization assays
(FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C and FIG.
6D). Since mammalian cells do not normally synthesize
either marker gene product, this provides direct evidence
that osteotomy repair cells were transfected *in vivo* and
then expressed the β-galactosidase and luciferase
transgenes as a functional enzymes.

## EXAMPLE IX
### ADENOVIRAL GENE TRANSFER INTO REGENERATING BONE *IN VIVO*

One of the alternative methods to achieve *in vivo*
gene transfer into regenerating bone is to utilize an
adenovirus-mediated transfer event. Successful
adenoviral gene transfer of a marker gene construct into
bone repair cells in the rat osteotomy model has been
achieved (FIG. 23A, FIG. 23B, and FIG. 23C).

The inventors employed the adenoviral vector pAd.
CMV*lacZ*, which is an example of a replication-defective
adenoviral vector which can replicate in permissive cells
(Stratford-Perricaudet *et al.*, 1992). In pAd.CMV*lacZ*,
the early enhancer/promoter of the cytomegalovirus (CMV)
is used to drive transcription of *lacZ* with an SV40
polyadenylation sequence cloned downstream from this
reporter (Davidson *et al.*, 1993).

The vector pAd.RSV4 is also utilized by the
inventors. This vector essentially has the same backbone
as pAdCMV*lacZ*, however the CMV promoter and the single
*Bgl*II cloning site have been replaced in a cassette-like
fashion with *Bgl*II fragment that consists of an RSV

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promoter, a multiple cloning site, and a poly(A⁺) site.
The greater flexibility of this vector is contemplated to
be useful in subcloning osteotropic genes, such as the
hPTH1-34 cDNA fragment, for use in further studies.

5

To generate recombinant PTH adenovirus, a 100-mm
dish of 293 cells is transfected using calcium phosphate
with 20 mg of a plasmid construct, e.g., the plasmid
containing the hPTH1-34 insert linearized with *NheI*, plus

10  2 mg of wild type adenovirus DNA digested with *XbaI* and
*ClaI*.  The adenovirus DNA is derived from adenovirus type
5, which contains only a single *XbaI* and *ClaI* sites and
has a partial deletion of the E3 region.  Approximately 7
days post-transfection, cells and media are harvested and

15  a lysate prepared by repeated freeze-thaw cycles.  This
lysate is diluted and used to infect 60 -mm dishes of
confluent 293 cells for 1 hour.  The cells are then
overlaid with 0.8% agar/1X MEM/2% calf serum/12.5 mM
MgCl₂.  Ten days post-infection, individual plaques are to

20  be picked and used to infect 60-mm dishes of 293 cells to
expand the amount of virus.  Positive plaques are
selected for further purification and the generation of
adenoviral stocks.

25       To purify recombinant adenovirus, 150-mm dishes of
75-90% confluent 293 cells are infected with 2-5
PFU/cell, a titer that avoids the potential cytotoxic
effects of adenovirus.  Thirty hours post-infection, the
cells are rinsed, removed from the dishes, pelleted, and

30  resuspended in 10 mM Tris-HCl, pH 8.1.  A viral lysate is
generated by three freeze-thaw cycles, cell debris is
removed by centrifugation for 10 min. at 2,000 rpm, and
the adenovirus is purified by density gradient
centrifugation.  The adenovirus band is stored at -20°C

35  in sterile glycerol/BSA until needed.

The solution of virus particles was sterilized and

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incubated with the implant material (from 6 min to overnight), and the virus-impregnated material was implanted into the osteotomy gap; where viral infection of cells clearly occurred. The results obtained clearly
5   demonstrated the exquisite specificity of the anti-β-gal antibody (Sambrook *et al.*, 1989), and conclusively demonstrated expression of the marker gene product in chondrocyte-like cells of the osteotomy gap. The nuclear-targeted signal has also been observed in pre-
10  osteoblasts.


## EXAMPLE X

### TRANSFER OF AN OSTEOTROPIC GENE
### STIMULATES BONE REGENERATION/REPAIR *IN VIVO*

15

In order for a parathyroid hormone (PTH) transgene to function as an osteotropic agent, it is likely that there is a requirement for the PTH/PTHrP receptor to be expressed in the bone repair tissue itself. Therefore,
20  the inventors investigated PTH/PTHrP receptor expression in the rat osteotomy model.

A Northern analysis of poly-A(+) RNA was conducted which demonstrated that the PTH/PHTrP receptor was
25  expression in osteotomy repair tissue (FIG. 13).

The inventors next investigated whether gene transfer could be employed to create transfected cells that constitutively express recombinant hPTH1-34 *in vivo*,
30  and whether this transgene can stimulate bone formation. The rate of new bone formation is analyzed as follows. At necropsy the osteotomy site is carefully dissected for histomorphometric analysis. The A-P and M-L dimensions of the callus tissue are measured using calipers.
35  Specimens are then immersion fixed in Bouins fixative, washed in ethanol, and demineralized in buffered formic acid. Plastic embedding of decalcified materials is used because of the superior dimensional stability of

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methacrylate during sample preparation and sectioning.

Tissue blocks are dehydrated in increasing alcohol
concentrations and embedded.  5 mm thick sections are cut
5     in the coronal plane using a Reichert Polycut microtome.
Sections are prepared from midway through the width of
the marrow cavity to guard against a sampling bias.
Sections for light microscopy are stained using a
modified Goldner's trichrome stain, to differentiate
10    bone, osteoid, cartilage, and fibrous tissue.  Sections
are cover-slipped using Eukitt's mounting medium
(Calibrated Instruments, Ardsley, NY).  Histomorphometric
analyses are performed under brightfield using a Nikon
Optiphot Research microscope.  Standard point count
15    stereology techniques using a 10 mm x 10 mm eyepiece grid
reticular are used.

Total callus area is measured at 125X magnification
as an index of the overall intensity of the healing
20    reaction.  Area fractions of bone, cartilage, and fibrous
tissue are measured at 250 X magnification to examine the
relative contribution of each tissue to callus formation.
Since the dimensions of the osteotomy gap reflect the
baseline (time 0), a measurement of bone area at
25    subsequent time intervals is used to indicate the rate of
bone infill.  Statistical significance is assessed using
analysis of variance, with post-hoc comparisons between
groups conducted using Tukey's studentized range t test.

30    In the 5-mm rat osteotomy model described above, it
was found that PTH transgene expression can stimulate
bone regeneration/repair in live animals (FIG. 6A, FIG.
6B, FIG. 6C, and FIG. 6D).  This is a particularly
important finding as it is known that hPTH1-34 is a more
35    powerful anabolic agent when given intermittently as
opposed to continuously, and it is the continuous-type
delivery that results from the gene transfer methods used

here.

Although the present inventors have already
demonstrated success of direct gene transfer into
5    regenerating bone *in vivo*, the use of *ex vivo* treatment
protocols is also contemplated.  In such embodiments,
bone progenitor cells would be isolated from a particular
animal or human subject and maintained in an *in vitro*
environment.  Suitable areas of the body from which to
10   obtain bone progenitor cells are areas such as the bone
tissue and fluid surrounding a fracture or other skeletal
defect (whether or not this is an artificially created
site) and from the bone marrow.  Isolated cells would
then be contacted with the DNA (or recombinant viral)
15   composition, with, or preferably without, a matrix, when
the cells would take up the DNA (or be infected by the
recombinant virus).  The stimulated cells would then be
returned to the site in the animal or patient where bone
repair is to be stimulated.
20

## EXAMPLE XI

### TRANSFER OF GENES TO ACHILLES' TENDON
### AND TO CRUCIATE LIGAMENT *IN VIVO*

25   The studies on regenerating bone described above
complement others by the inventors in which gene transfer
was successfully employed to introduce genes into
Achilles' tendon (FIG 3A, FIG. 3B, FIG. 3C, FIG. 3D, and
FIG. 3E) and cruciate ligament (FIG. 4).
30

The Achilles' tendon consist of cells and
extracellular matrix organized in a characteristic tissue
architecture.  Tissue wounding can disrupt this
architecture and stimulate a wound healing response.  The
35   wounded tendon will regenerate, as opposed to scar, if
its connective tissue elements remain approximately
intact.  Regeneration is advantageous because scar tissue
is not optimally designed to support normal mechanical

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function. Segmental defects in tendon due to traumatic injury may be treated with biological or synthetic implants that encourage neo-tendon formation. This strategy is limited, however, by the availability of effective (autologous) biological grafts, the long term stability and compatibility of synthetic prostheses, and the slow rate of incorporation often observed with both types of implants.

The inventors hypothesized that the effectiveness of biological grafts may be enhanced by the over-expression of molecules that regulate the tissue regeneration response. Toward this end, they developed a model system in which segmental defects in Achilles' tendon are created and a novel biomaterial, is used as a tendon implant/molecular delivery agent. In the present example, the ability to deliver and express marker gene constructs into regenerating tendon tissue is demonstrated.

Plasmid (pSVβgal, Promega) stock solutions were prepared according to standard protocols (Sambrook et al., 1989). SIS graft material was prepared from a segment of jejunum of adult pigs (Badylak et al., 1989). At harvest, mesenteric tissues were removed, the segment was inverted, and the mucosa and superficial submucosa were removed by a mechanical abrasion technique. After returning the segment to its original orientation, the serosa and muscle layers were rinsed, sterilized by treatment with dilute peracetic acid, and stored at 4°C until use.

Mongrel dogs (all studies) were anesthetized, intubated, placed in right-lateral recumbency upon a heating pad, and maintained with inhalant anesthesia. A lateral incision from the musculotendinous junction to the plantar fascia was used to expose the Achilles'

tendon. A double thickness sheet of SIS was wrapped
around a central portion of the tendon, both ends were
sutured, a 1.5 cm segment of the tendon was removed
through a lateral opening in the graft material, and the
5      graft and surgical site were closed. The leg was
immobilized for 6 weeks and then used freely for 6 weeks.
Graft tissues were harvested at time points indicated
below, fixed in Bouins solution, and embedded in
paraffin. Tissue sections (8 $\mu$m) were cut and used for
10     immunohistochemistry.

In an initial study, SIS material alone (SIS-alone
graft) engrafted and promoted the regeneration of
Achilles' tendon following the creation of a segmental
15     defect in mongrel dogs as long as 6 months post surgery.
The remodeling process involved the rapid formation of
granulation tissue and eventual degradation of the graft.
Scar tissue did not form, and evidence of immune-mediated
rejection was not observed.
20

In a second study, SIS was soaked in a plasmid DNA
solution (SIS+plasmid graft) and subsequently implanted
as an Achilles' tendon graft (n=2 dogs) or a cruciate
ligament graft (n=2 dogs) in normal mongrel dogs. A
25     pSV$\beta$gal plasmid that employs simian virus 40 regulatory
sequences to drive $\beta$-galactosidase ($\beta$-gal) activity was
detectable by immunohistochemistry using a specific
antibody in 4/4 animals. As a negative control, $\beta$-gal
activity was not detected in the unoperated Achilles'
30     tendon and cruciate ligament of these animals. It
appeared, therefore, that SIS facilitated the uptake and
subsequent expression of plasmid DNA by wound healing
cells in both tendon and ligament.

35     A third study was designed to evaluate the time
course of $\beta$-gal transgene expression. SIS + plasmid
grafts were implanted for 3, 6, 9, and 12 weeks (n=2 dogs

pr time point) and transgene expression was assayed by
immunohistochemistry and by *in situ* hybridization.
Cross-sections (8-$\mu$m) of Bouins fixed, paraffin embedded
tissue were cut and mounted on ProbeOn Plus slides

5  (Fisher). Immunohistochemistry was performed according
to the protocol provided with the Histostain-SP kit
(Zymed). In brief, slides were incubated with a well
characterized anti-$\beta$-galactosidase antibody (1:200
dilution, 5'$\rightarrow$3'), washed in PBS, incubated with a

10  biotinylated second antibody, washed, stained with the
enzyme conjugate plus a substrate-chromogen mixture, and
then counterstained with hematoxylin and eosin.

Bacterial $\beta$-gal activity was detected in tendons

15  that received the SIS+plasmid graft (8/8 animals).
Although not rigorously quantitative, transgene
expression appeared to peak at 9-12 weeks. Bacterial
$\beta$-gal gene expression was not detected in animals that
received SIS-alone grafts (N=2, 3 weeks and 12 weeks).

20  Again, scar tissue did not form and evidence of immune-
mediated rejection was not observed.

This study demonstrated that the mucosal biomaterial
SIS can function as an autologous graft that promotes the

25  regeneration of tissues such as Achilles' tendon and
anterior cruciate ligament. SIS can also be used to
deliver a marker gene construct to regenerating tissue.

**EXAMPLE XIII**

30  **MECHANICAL PROPERTIES OF NEW BONE FORMATION**

The mechanical properties of new bone formed during
gene transfer may be measured using, *e.g.*, whole bone
torsion tests which create a stress state in which the

35  maximum tensile stresses will occur on planes that lie
obliquely to the bone's longitudinal axis. Such tests
may provide important inferences about the mechanical

anisotropy of callus tissue and the degree of osseous
integration of new bone tissue. These tests are
particularly advantageous in the evaluation of fracture
specimens, e.g., the irregular shape of callus tissue
5    typically precludes the use of whole bone 4-point bending
tests because it is impossible to reproducibly align the
points from specimen to specimen.

Femurs are tested on an MTS Servohydraulic Testing
10   Machine while moist and at room temperature. A torque
sensor and rotary variable displacement transduces
provides data for torque-angular displacement curves.
Specially designed fixtures support each bone near the
metaphyseal-diaphyseal junctions, and apply a 2-point
15   load to the diaphysis. Tests are conducted at a constant
rate of displacement equal to 20 degrees/sec. A 250
inch-ounce load cell measures the total applied force.
All bones are tested while moist and room temperature.
Torque and angular displacement data are acquired using
20   an analog-to-digital converter and a Macintosh computer
and software. From this data, the following variables
are calculated: a) maximum torque, b) torsional
stiffness, the slope of the pre-yield portion of the
curve determined from a linear regression of the data, c)
25   energy to failure, the area under the torque-angular
displacement curve to the point of failure, and d) the
angular displacement ratio, the ratio of displacement at
failure to displacement at yield. Statistical
significance is determined Analysis of Variance followed
30   by multiple comparisons with appropriate corrections
(e.g., Bonferroni).

This invention also provides a means of using
osteotropic gene transfer in connection with
35   reconstructive surgery and various bone remodelling
procedures. The techniques described herein may thus be
employed in connection with the technology described by

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Yasko *et al.*, 1992; Chen *et al.*, 1991; and Beck *et al.*, 1991, each incorporated herein by reference.

### EXAMPLE XIV
### TYPE II COLLAGEN PROMOTES NEW BONE GROWTH

Certain matrix materials are capable of stimulating at least some new growth in their own right, *i.e.*, are "osteoconductive materials". Potential examples of such materials are well known in the field of orthopedic research and include preparations of hydroxyapatite; preparations of crushed bone and mineralized collagen; PLGA block copolymers and polyanhydride. The ability of these materials to stimulate new bone formation distinguishes them from inert implant materials such as methylcellulose, which have in the past been used to deliver BMPs to sites of fracture repair.

This Example relates to a study using the rat osteotomy model with implants made of collagen type I (Sigma), collagen type II (Sigma), and UltraFiber™ (Norian Corp.). These materials have been placed *in situ* without DNA of any type. Five animals received an osteotomy with 10 mg of a type II collagen implant alone (10 mg refers to the original quantity of lyophilized collagen). Five of five control animals received an osteotomy with 10 mg of a type I collagen implant alone. Animals were housed for three weeks after surgery and then sacrificed.

The results of these studies were that SIS appeared to retard new bone formation; type I collagen incited a moderately intense inflammatory response; and UltraFiber™ acted as an osteoconductive agent. The type II collagen implant studies yielded surprising results in that 10 mg of this collagen was found to promote new bone formation in the 5-mm osteotomy model (FIG. 22A, FIG. 22B, and FIG.

22C).  New bone - bridging the osteotomy gap - was
identified three weeks after surgery in 5/5 animals that
received a type II collagen implant alone (i.e., minus
DNA of any type).  In contrast, fibrous granulation
5    tissue, but no evidence of new bone formation, was
obtained in 5/5 animals receiving a type I collagen
implant alone.


Radiographic analysis demonstrated conclusively that
10    all animals receiving an osteotomy with a type II
collagen implant without exception showed radio-dense
material in the osteotomy gap (FIG. 22A).  In sharp
contrast, radiographic analysis of all animals receiving
a type I collagen implant revealed no radio-dense
15    material forming in the osteotomy gap (FIG. 22B).  The
arrow in FIG. 22A point to the new bone growth formed in
the osteotomy gap of type II collagen implanted-animals.
No such new bone growth was observed in the animals
receiving type I collagen implants (FIG. 22B).
20

FIG. 22C demonstrates the results of the osteotomy
with a type II collagen implant.  The arrow points to the
area of new bone formed in the osteotomy gap.  In
contrast, only fibrous granulation tissue was identified
25    in the type I collagen gap.


Previous studies have suggested that type II
collagen plays only a structural role in the
extracellular matrix.  The results of the type II
30    collagen implant studies are interesting because they
demonstrate a novel and osteoconductive role for type II
collagen during endochondral bone repair.  To further
optimize the osteoconductive potential of type II
collagen, a yeast expression vector that encodes for type
35    II collagen (full length $\alpha 1$(II) collagen) will be
employed to produce recombinant $\alpha 1$(II) collagen protein.

EXAMPLE XV

IDENTIFICATION OF FURTHER OSTEOTROPIC GENES:

ISOLATION OF A NOVEL LATENT TGF-$\beta$
BINDING PROTEIN-LIKE (LTBP-3) GENE

The TGF-$\beta$s represent a family of structurally
related molecules with diverse effects on mammalian cell
shape, growth, and differentiation (Roberts and Sporn,
1990). Initially synthesized as a precursor consisting
of an amino-terminal propeptide followed by mature TGF-$\beta$,
two chains of nascent pro-TGF-$\beta$ associate in most tissues
to form a Mr ~106,000 inactive disulfide-bonded dimer.
Homodimers are most common, but heterodimers have also
been described (Cheifetz et al., 1987; Ogawa et al.,
1992). During biosynthesis the mature TGF-$\beta$ dimer is
cleaved from the propeptide dimer. TGF-$\beta$ latency results
in part from the non-covalent association of propeptide
and mature TGF-$\beta$ dimers (Pircher et al., 1984 and 1986;
Wakefield et al., 1987; Millan et al., 1992; see also
Miyazono and Heldin, 1989). Consequently, the propeptide
dimer is often referred to as the latency associated
protein (LAP), and LAP plus the disulfide-bonded TGF-$\beta$
dimer are also known as the small latent complex. In the
extracellular space small latent complexes must be
dissociated to activate mature TGF-$\beta$. The mechanism of
activation of the latent complex is thought to be one of
the most important steps governing TGF-$\beta$ effects (Lyons
et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et
al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent
growth factor complexes may contain additional high
molecular weight proteins. The best characterized of
these high molecular weight proteins is the latent TGF-$\beta$
binding protein, or LTBP (Miyazono et al., 1988; Kanzaki
et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992;
Taketazu et al., 1994). LTBP produced by different cell

types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990).

5 Latent TGF-β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF-β, but rather it is linked by a disulfide bond to LAP.

10 Two LTBPs have been isolated to date. The deduced human LTBP-1 amino acid sequence is comprised of a signal peptide, 16 epidermal growth factor-like repeats with the potential to bind calcium (EGF-CB repeats), 2 copies of a unique motif containing 8 cysteine residues, an RGD cell

15 attachment motif, and an 8 amino acid motif identical to the cell binding domain of the laminin B2 chain (Kanzaki et al., 1990). There is evidence that LTBP-1 binds calcium, which, in turn, induces a structural change that protects LTBP from proteolytic attack (Colosetti et al.,

20 1993). LTBP-2 shows 41% sequence identity to LTBP-1, and its structural domains show a similar overall organization (Moren et al., 1994).

While the functions of LTBP-1 and LTBP-2 presently

25 are unknown, several ideas have been put forward in the literature. First, LTBP may regulate the intracellular biosynthesis of latent TGF-β precursors. Cultured erythroleukemia cells efficiently assemble and secrete large latent TGF-β complexes, whereas they slowly secrete

30 small latent TGF-β complexes that contain anomalous disulfide bonds (Miyazono et al., 1991; Miyazono et al., 1992). Therefore, LTBP may facilitate the normal assembly and secretion of latent TGF-β complexes. Second, LTBP may target latent TGF-β to specific types of

35 connective tissue. Recent evidence suggests that the large latent TGF-β complex is covalently bound to the extracellular matrix via LTBP (Taipale et al., 1994).

Based on these observations, LTBP has been referred to as
a "matrix receptor", *i.e.* a secreted protein that targets
and stores latent growth factors such as TGF-$\beta$ to the
extracellular matrix.   Third, LTBP may modulate the
5       activation of latent complexes.   This idea is based in
part on recent evidence which suggests that mature TGF-$\beta$
is released from extracellular storage sites by proteases
such as plasmin and thrombin and that LTBP may protect
small latent complexes from proteolytic attack (Falcone
10      *et al.*, 1993; Benezra *et al.*, 1993; Taipale *et al.*,
1994), *i.e.* protease activity may govern the effect of
TGF-$\beta$ in tissues, but LTBP may modulate this activity.
Fourth, LTBP may plays an important role in targeting
the latent TGF-$\beta$ complex to the cell surface, allowing
15      latent TGF-$\beta$ to be efficiently activated (Flaumenhaft *et
al.*, 1993).


**A.   MATERIALS AND METHODS**


20      **1.   cDNA Cloning**


Aliquots (typically 40-50,000 PFU) of phage
particles from a cDNA library in the $\lambda$ZAPII® vector made
from NIH 3T3 cell mRNA (Stratagene) and fresh overnight
25      XL1-Blue™ cells (grown in Luria broth supplemented with
0.4% maltose in 10 mM MgSO$_4$) were mixed, incubated for 15
min. at 37°C, mixed again with 9 ml of liquid (50°C) top
layer agarose (NZY broth plus 0.75% agarose), and then
spread evenly onto freshly poured 150 mm NZY-agar plates.
30      Standard methods were used for the preparation of
plaque-lifts and filter hybridization (42°C, in buffer
containing 50% formamide, 5X SSPE, 1X Denhardt's, 0.1%
SDS, 100 mg/ml salmon sperm DNA, 100 mg/ml heparin).
Filters were washed progressively to high stringency
35      (0.1X SSC/0.1% SDS, 65°C).   cDNA probes were radiolabeled
by the nick translation method using commercially
available reagents and protocols (Nick Translation Kit,

Boehringer Mannheim). Purified phage clones were
converted to pBluescript® plasmid clones, which were
sequenced using Sequenase (v2.0) as described (Chen et
al., 1993; Yin et al., 1995). Sequence alignment and
5  identity was determined using sequence analysis programs
from the Genetics Computer Group (MacVector).


### 2.  Tissue *In Situ* Hybridization


10     To prepare normal sense and antisense probes, a
unique 342 bp fragment from the 3' untranslated region
(+3973 to +4314, counting the "A" of the initiator Met
codon as +1; see "ish", Fig. 1) was subcloned into the
pBSKS+ plasmid (Stratagene, Inc.). Template DNA was
15  linearized with either *Eco*RI or *Bam*HI, extracted, and
precipitated with ethanol. Sense and antisense
transcripts were generated from 1 mg template with T3 and
T7 polymerases in the presence of [$^{35}$S]UTP at >6 mCi/ml
(Amersham, >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega),
20  with the remaining *in vitro* transcription reagents
provided in a kit (SureSite, Novagen Inc.). After
transcription at 37°C for 1 h, DNA templates were removed
by a 15 min. digest at 37°C with 0.5 U/ml RNase-free
DNase I, extracted, and precipitated with ethanol.
25  Riboprobes were hydrolyzed to an average final length of
150 bp by incubating in 40 mM NaHCO$_3$, 60 mM Na$_2$CO$_3$, 80 mM
DTT for ~40 min. at 60°C. Hydrolysis was terminated by
addition of sodium acetate, pH 6.0, and glacial acetic
acid to .09 M and 0.56% (v/v), respectively, and the
30  probes were then ethanol precipitated, dissolved in 0.1 M
DTT, counted, and stored at -20°C until use. Day
8.5-9.0, day 13.5, and day 16.5 mouse embryo tissue
sections (Novagen) and the *in situ* hybridization protocol

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were exactly as described (Chen *et al.*, 1993; Yin *et al.*, 1995).

### 3.  Northern Analysis

MC3T3-E1 cell poly(A+) RNA (2-10 mg aliquots) was electrophoresed on a 1.25% agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane (Hybond-N, Amersham).  The RNA was cross-linked to the membrane by exposure to a UV light source ($1.2 \times 10^6$ mJ/cm$^2$, UV Stratalinker 2400, Stratagene) and then pre-hybridized for >15 min. at 65°C in Rapid-Hyb buffer (Amersham, Inc.).  A specific cDNA probe consisting solely of untranslated sequence from the 3' end of the transcript was $^{32}$P-labeled by random priming and used for hybridization (2 h at 65°C).  Blots were washed progressively to high stringency (0.1X SSC/0.1% SDS, 65°C), and then placed against x-ray film with intensifying screens (XAR, Kodak) at -86°C.

### 4.  Antibody Preparation

LTBP-3 antibodies were raised against a unique peptide sequence found in domain #2 (amino acids 155-167).  Peptide #274 (GESVASKHAIYAVC) (SEQ ID NO:16) was synthesized using an ABI model 431A synthesizer employing FastMoc chemistry.  The sequence was confirmed using an ABI473 protein sequencer.  A cysteine residue was added to the carboxy-terminus to facilitate crosslinking to carrier proteins.  For antibody production, the synthetic peptide was coupled to rabbit serum albumin (RSA) using MBS (*m*-maleimidobenzoic acid-N-hydroxysuccinimide ester) at a substitution of 7.5 mg peptide per mg of RSA.  One mg of the peptide-RSA conjugate in 1 ml of Freund's complete adjuvant was injected subcutaneously at 10 different sites along the backs of rabbits.  Beginning at 3 weeks after initial

immunization, the rabbits were given bi-weekly booster
injections of 1 mg peptide-RSA in 100 ul of Freund's
incomplete adjuvant.  IgG was prepared by mixing immune
serum with caprylic acid (0.7 ml caprylic acid per ml
5    serum), stirring for 30 min., and centrifuging at 5,000 x
g for 10 min.  The supernatant was decanted and dialyzed
against two changes of phosphate buffered saline (PBS)
overnight at 4°C.  The antibody solution was then
affinity purified by passing it over a column containing
10   the immunizing peptide coupled to Affi-gel 10 affinity
support.  Bound antibodies were eluted with 0.2 M glycine
(pH 2.3), immediately dialyzed against PBS, and
concentrated to 1 mg/ml.  prior to storage at -70°C.

15       5.   **Transfection**

     Transient transfection was performed using standard
protocols (Sambrook et al., 1989).  Briefly, subconfluent
cells (covering ~20% of a 100 mm plastic tissue culture
20   dish) were washed 2x in DMEM tissue culture medium
(GIBCO) and then incubated for 3 hrs. at 37°C in a
sterile mixture of DEAE-dextran (0.25 mg/ml), chloroquine
(55 mg/ml), and 15 mg plasmid DNA (Courey and Tjian,
1988).  Cells then were shocked by incubation with 10%
25   DMSO in sterile PBS for 2 min. at 37°C, washed 2x with
DMEM (Sambrook et al., 1989), and incubated in DMEM plus
10% fetal calf serum and antibiotics for 72 hr. at 37°C.

     6.   **Immunoprecipitation**
30

     For immunoprecipitation, 1 ml of antibody (1:400
final concentration, in PBS-TDS buffer: 0.38 mM NaCl, 2.7
mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 1% Triton X-100,
0.5% Deoxycholic acid, and 0.1% SDS) was added to 1 ml of
35   radiolabeled medium proteins.  The mixture was incubated
with shaking at 4°C for 1 hr., protein A-sepharose CL-4B
beads were added (200 ml, 10% suspension), and this

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mixture was incubated with shaking for one additional
hour at 4°C.  Immunoprecipitated proteins were pelleted
by brief centrifugation, the pellet was washed 6x with
PBS-TDS buffer, 2x protein loading dye was added, and the
5     samples were boiled for 5 min. and then fractionated on
4-18% gradient SDS-PAGE (Bonadio et al., 1985).  Cold
molecular weight markers (200 kDa-14.3 kDa, Rainbow mix,
Amersham) were used to estimate molecular weight.  The
gel was dried and exposed to film for the indicated time
10    at room temperature.


### 7. Western Analysis

Fractionated proteins within SDS-polyacrylamide gels
15    were transferred to a nitrocellulose filter for 2 hours
using Tris-glycine-methanol buffer, pH 8.3 at 0.5 mA/cm$^2$.
The filter was blocked, incubated with nonfat milk plus
antibody (1:1000 dilution) for 2 hr, and washed.
Antibody staining was visualized using the ECL Western
20    blotting reagent (Amersham) according to the
manufacturer's protocols.


### B. RESULTS

25    In this study, the inventors isolated and
characterized a novel murine fibrillin-like cDNA encoding
LTBP-3.  To clone the murine LTBP-3 gene, cDNA from a 3T3
cell cDNA library was amplified using human fibrillin-1
PCR™ primers under low stringency conditions (i.e.,
30    annealing at 37°C initially for 10 cycles, followed by
annealing at 60°C for 30 cycles).  The results indicated
that a murine DNA fragment of unexpectedly low homology
(~50%) to human fibrillin-1 was obtained.  Molecular
cloning of the authentic murine fibrillin-1 transcript
35    was also performed, confirming the human and murine
fibrillin-1 coding sequences share >95% sequence
identity.  The murine fibrillin-1 and PCR™ sequences were

different, which suggested that the PCR™ product may have
been derived from a related, fibrillin-like cDNA. The
3T3 cell cDNA library was screened at high stringency
using the murine PCR™ product as the probe in order to
5   test this hypothesis. A cDNA walking strategy eventually
yielded seven overlapping cDNA clones (FIG. 14). It
provides a unique mRNA of 4,314 nucleotides, with an open
reading frame of 3,753 nucleotides (SEQ ID NO:2). The
deduced molecule is a unique polypeptide of 1,251 amino
10  acids (SEQ ID NO:3). Excluding the signal peptide (21
amino acids), the novel fibrillin-like molecule consists
of five structurally distinct regions (Region 1- Region
5), and although similar to murine fibrillin-1 (FIG.
15A), its domain structure is unique as is evidenced by
15  the schematic representation of LTBP-3 shown in FIG. 15B.

Domain #1 is a 28 amino acid segment with a net
basic charge (est. pI, 12.36) that may allow for binding
acidic molecules in the extracellular matrix (e.g.,
20  acidic proteoglycans). Sequences rich in basic amino
acids may also function as endoproteolytic processing
signals (Barr, 1991; Steiner et al., 1992), which
suggests that the NH$_2$-terminus may be proteolytically
processed. Domain #2 extends for of 390 amino acids,
25  consisting of an EGF-like repeat, a 135 amino acid
segment that was proline-rich (20.7%) and glycine-rich
(11.8%) but not cysteine-rich, a Fibmotif (Pereira
et al., 1993), an EGF-CB repeat, and a TGF-bp repeat.
Domain #3 is a 113 amino acid segment characterized by
30  its high proline content (21%). Domain #4 extends for
678 amino acids and consists of 14 consecutive cysteine-
rich repeats. Based on structural homologies, 12/14
repeats were epidermal growth factor-calcium binding
(EGF-CB) motifs (Handford et al., 1991), whereas 2/14
35  were transforming growth factor-$\beta$-binding protein (TGF-
bp) motifs (Kanzaki et al., 1990). Finally, domain #5 is
a 22 amino acid segment at the carboxy-terminus. The

conceptual amino acid sequence encoded by the open
reading frame consisted of 1,251 amino acids (FIG. 15B)
with an estimated pI of 5.92, a predicted molecular mass
of 134,710 Da, and five potential $N$-linked glycosylation
5   sites.  No RGD sequence was present.


    Northern blot analysis of murine embryo RNA using a
3' untranslated region probe identified a transcript band
of ~4.6 kb.  In this regard, 4,310 nt have been isolated
10  by cDNA cloning, including a 3' untranslated region of
401 nt and a 5' upstream sequence of 156 nt.  The
apparent discrepancy between the Northern analysis result
and the cDNA sequence analysis suggested that the 5'
upstream sequence may include ~300 nt of additional
15  upstream sequence.  This estimate was consistent with
preliminary primer extension mapping studies indicating
that the 5' upstream sequence is 400-500 nt in length.


    A total of 19 cysteine-rich repeats were found in
20  domains #2 and #4 of the murine LTBP-like (LTBP-3)
polypeptide.  Thirteen were EGF-like and 11/13 contained
the calcium binding consensus sequence.  This consensus
was derived from an analysis of 154 EGF-CB repeats in 23
different proteins and from structural analyses of the
25  EGF-CB repeat, both bound and unbound to calcium ion
(Selander-Sunnerhagen et al., 1992).  Variations on the
consensus have been noted previously and one of these, D-
L-N/D-E-$C_1$, was identified in the third EGF-like repeat of
domain #4.  In addition, a potential calcium binding
30  sequence which has not previously been reported (E-T-N/D-
E-$C_1$) was identified in the first EGF-like repeat of
domain #4.  Ten of thirteen EGF-CB repeats also contained
a second consensus sequence which represents a
recognition sequence for an Asp/Asn hydroxylase that co-
35  and post-translationally modifies D/N residues (Stenflo
et al., 1987; Gronke et al., 1989).

Although about one-half the size, the deduced
polypeptide was organized like fibrillin-1 in that it
consisted of a signal peptide followed by 5 structurally
distinct domains, *i.e.*, two domains with numerous EGF-
5    like, EGF-CB and Fib repeats and a third with a proline-
rich sequence (Pereira *et al.*, 1993). However,
comparison of each of these domains using the GAP and
BESTFIT programs (Genetics Computer Group) has revealed a
low level of amino acid homology of only 27% over the
10    five structural domains shared by the deduced murine
polypeptide and human fibrillin-2. These values are low
for a putative fibrillin family member because fibrillin-
1 and fibrillin-2 share ~50% identity (Zhang *et al.*,
1994).

15

A search of available databases revealed that the
deduced murine polypeptide was most similar to the human
and rat latent TGF-β binding proteins (Kanzaki *et al.*,
1990; Tsuji *et al.*, 1990). In this regard LTBP was found
20    to be similar to fibrillin in that it could also be
divided into five structurally distinct domains (FIG.
15A, FIG. 15B, and FIG. 15C). These include a relatively
short domain downstream of the signal peptide with a net
basic charge (amino acids 21-33, est. pI, 11.14); a
25    domain consisting of EGF-like, EGF-CB, TGF-bp, and Fib
motifs plus a proline-rich and glycine-rich sequence
(amino acids 34-407); a proline-rich domain (amino acids
408-545); a large, domain consisting of EGF-CB, TGF-bp,
and TGF-bp-like repeat motifs (amino acids 546-1379); and
30    a relatively short domain at the carboxy terminus (amino
acids 1380-1394). Amino acid sequence comparison of the
deduced murine and human polypeptides shows 60% identity
for domain #1, 52% identity for domain #2, 30% identity
for domain #3, 43% identity for domain #4, and 7%
35    identity for domain #5. The average identity over the
five domains shared by the murine polypeptide and human

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cardiovascular tissue (myocardium plus endocardium) was also observed.

Microscopy of day 13.5 and day 16.5 embryos demonstrated expression of the murine gene by skeletal muscle cells and by cells involved in intramembranous and endochondral bone formation. The transcript was expressed by osteoblasts and by periosteal cells of the calvarium, mandible and maxilla. The transcript was also identified in both cartilage and bone of the lower extremity. A positive signal was detected in perichondrial cells and chondrocytes (proliferating > mature > hypertrophic) of articular cartilage, the presumptive growth plate, and the cartilage model within the central canal. The positive signal was also expressed by blood vessel endothelial cells within the mid-diaphysis, and the surrounding muscle cells (FIG. 18A, FIG. 18B, FIG. 18C, FIG. 18D, FIG. 18E, FIG. 18F, FIG. 19G, FIG. 18H, FIG. 18I, FIG. 18J, FIG. 18K, FIG. 18L, FIG. 18M, FIG. 18N, FIG. 18O, and FIG. 18P).

Respiratory epithelial cells lining developing small airways and connective tissue cells in the pulmonary interstitium expressed the murine transcript, as did myocardial cells (atria and ventricles) and endocardial cushion tissue. Cells within the walls of large arteries also expressed the transcript. Expression of the murine gene was identified in several organs of the alimentary system, including the tongue, esophagus, stomach, small and large intestine, pancreas and liver. Mucosal epithelial cells lining the upper and lower digestive tract plus the smooth muscle and connective tissue cells found in the submucosa expressed the transcript, as did acinar cells of the exocrine pancreas. Despite the high level of transcript expression in the liver, these results suggest both cell populations express the LTBP-3 transcript.

In the kidney, expression above the basal level was observed in cells of developing nephrons, the ureteric bud, kidney blastema and the kidney interstitium. In the skin, epidermal and adnexal keratinocytes, dermal

5    connective tissue cells, and brown fat cells within the dorsal subcutis expressed the murine transcript. In the central and peripheral nervous systems, ganglion cells within the cerebrum, brainstem, spinal cord, and peripheral nerves expressed the murine transcript. The

10   transcript was also intensely expressed by cells of the developing murine retina.

Thus, the murine gene is widely expressed by both epithelial and connective tissue cells, a pattern that

15   would be expected for a latent TGF-$\beta$ binding protein. Three final observations argue that the LTBP-like (LTBP-3) sequence presented in FIG. 25 is not simply the murine homologue of human LTBP. First, domain #4 of the murine LTBP-like (LTBP-3) sequence has a smaller number of EGF-

20   like repeat motifs than human and rat LTBP (8 versus 11). Second, portions of the human and rat LTBP-like coding sequence were characterized and found to share ~90% identity with human and rat LTBP but only 65% identity with the murine LTBP-like gene. Third, the human LTBP

25   and LTBP-like genes are localized to separate chromosomes. Human LTBP was assigned to human chromosome 2 based on the analysis of human $x$ rodent somatic cell hybrid lines (Stenman et al., 1994). The present invention represents the first mapping of an LTBP gene in

30   the murine. The human LTBP-like genes was recently localized to chromosome 11 band q12, while the murine gene was mapped to murine chromosome 19, band B (a region of conserved synteny), using several independent approaches, including fluorescent in situ hybridization.

35

The first indication of alternative splicing came from molecular cloning studies in the murine, in which

independent cDNA clones were isolated with a deletion of
51 bp from the coding sequence. PCR™/Southern blot
analysis provided additional evidence that the homologous
51 bp sequence was alternatively spliced in normal murine
5    embryo tissues.

Northern blot analysis also demonstrated that the
novel fibrillin gene was also expressed in rat callus
three weeks after osteotomy, after mineralization has
10   begun. Gene expression in normal adult rat bone tissue
was insignificant, which suggests that microfibrils are
an important part of the bone fracture healing response.
The novel fibrillin-like gene was expressed in callus as
a pair of alternatively spliced transcripts. This result
15   has been independently reproduced on three occasions.
Molecular cloning of the novel fibrillin gene in both
murine and rat has identified potential splice junction
sites for the alternative splicing event.

20    MC3T3-E1 murine pre-osteoblasts were used to
demonstrate that the murine gene product was capable of
binding TGF-$\beta$. MC3T3-E1 cells were utilized because they
synthesize and secrete TGF-$\beta$, which may act as an
autocrine regulator of osteoblast proliferation (Amarnani
25   et al., 1993; Van Vlasselaer et al., 1994; Lopez-Casillas
et al., 1994).

To determine whether or not MC3T3-E1 cells
co-expressed the murine gene product of TGF-$\beta$, cells were
30   plated on 100-mm dishes under differentiating conditions
(Quarles et al., 1992) and the medium was replaced twice
weekly. Parallel dishes were plated and assayed for cell
number and alkaline phosphatase activity, which confirmed
that osteoblast differentiation was indeed taking place.
35   Equal aliquots of total cellular RNA was prepared from
these MC3T3-E1 cells after 5, 14 and 28 days in culture
for Northern blot analysis. As shown in FIG. 19,

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expression of the new murine gene peaked on day 14 of culture.  Since MC3T3-E1 cells also show a peak in alkaline phosphatase activity on day 14 of culture (Quarles *et al.*, 1992), the results suggest for the first
5    time that LTBP-2 gene expression is an early marker of osteoblast differentiation.

## C. DISCUSSION

10        This study reports the molecular cloning of a novel LTBP-like gene that contains numerous EGF-like repeats. Northern analysis indicates that the gene encodes a single transcript of ~4.6 kb in murine embryo tissues. The deduced amino acid sequence of the murine gene
15    product appears to be a secreted polypeptide of 1,251 amino acids.  Although it is similar to fibrillin, the overall structural organization and expression pattern of this gene product most resembles LTBP, a latent TGF-$\beta$ binding protein that was originally isolated and
20    characterized by Heldin and co-workers (Kanzaki *et al.*, 1990).  Several observations strongly suggest that LTBP and the murine LTBP-like gene product are therefore derived from related but distinct genetic loci.  First, LTBP and the LTBP-like coding sequence share ~40%
25    identity and differences exist in the number of EGF-CB repeats in the deduced polypeptide sequence of the two molecules.  Second, a portion of the murine LTBP gene has been cloned and shown to share ~90% identity with human and rat LTBP.  Conversely, portions of the human and rat
30    LTBP-like genes have been cloned and shown to share ~90% identity with the murine LTBP-like gene.  Third, LTBP and the LTBP-like gene reside on different human chromosomes (Stenman *et al.*, 1994).  Taken together, these data suggest that a family of at least two LTBP genes exists.
35

         Similarities in the structural organization of LTBP-1 and the fibrillin-1 and fibrillin-2 polypeptides have

been noted previously (Pereira *et al.*, 1993; Zhang
*et al.*, 1994; Taipale *et al.*, 1994). For example, LTBP-1
and the fibrillins are all secreted extracellular matrix
constituents. Moreover, each polypeptide can be
5    organized into five domains, two of which consists
predominantly of EGF-CB and TGF-bp repeat motifs. LTBP-1
and fibrillin-1 also share a domain that is proline-rich,
and LTBP possesses an 8-cysteine repeat previously
referred to as the "Fib motif" because it was assumed to
10   be unique to fibrillin (Pereira *et al.*, 1993). These
similarities likely explain the initial isolation and
cloning of the LTBP-2 PCR™ product, especially since the
human oligonucleotide primers used to initially amplify
murine cDNA were designed to direct the synthesis of an
15   EGF-CB repeat in domain #4.

Another point of distinction between LTBP-2 and
fibrillin concerns the spacing of conserved cysteines C4
and C5 in EGF-like repeats. Fibrillin-1 and fibrillin-2
20   each contain >50 such repeats, and in every one the
spacing is $C_4$-X-$C_5$. While this pattern is repeated in a
majority of the EGF-like repeats in LTBP-1 and LTBP-2,
both genes also contain repeats with the spacing $C_4$-X-X-
$C_5$. Although the significance of this observation is
25   unclear, variation in the number of amino acids between $C_4$
and $C_5$ would not be expected to alter the function of the
EGF-like repeat. Mature EGF is a 48 amino acid secreted
polypeptide consisting of two subdomains that have few
interdomain contacts (Engel, 1989; Davis, 1990). The
30   larger $NH_2$-terminal subdomain consists of residues 1-32
and is stabilized by a pair of disulfide bonds ($C_1$-$C_3$ and
$C_2$-$C_4$), whereas the smaller COOH-terminal subdomain (amino
acids 33-48) is stabilized by a single disulfide bond ($C_5$-
$C_6$). The COOH-terminal subdomain has a highly conserved
35   conformation that only is possible if certain residues
and the distances between them are well conserved, while
conformation-sequence requirements for the NH2-terminal

subdomain are relatively relaxed. Variation in $C_4$-$C_5$ spacing would not be expected to alter conformation because these residues do not normally form a disulfide bond and the spacing variation occurs at the interface of

5 subdomains that would not be predicted to interact. The cloning of additional genes will decide whether variation in $C_4$-$C_5$ spacing is a reliable discriminator between members of the LTBP and fibrillin gene families.

10 The LTBP-2 gene is expressed more widely during development than fibrillin-1 or fibrillin-2. Studies in developing murine tissues have shown that the *Fbn-1* gene is expressed by mesenchymal cells of developing connective tissue, whereas the murine LTBP-like gene is

15 intensely expressed by epithelial, parenchymal and stromal cells. Earlier reports have suggested that TGF-$\beta$ plays a role in differentiation and morphogenesis during murine development (Lyons and Moses, 1990), when TGF-$\beta$ is produced by epithelial, parenchymal and stromal cells.

20 Tsuji *et al.*, (1990) and others have suggested that the expression of TGF-$\beta$ binding proteins should mirror that of TGF-$\beta$ itself; the expression pattern of the LTBP-2 gene over the course of murine development is consistent with this expectation. However, the LTBP-2 gene may not

25 be completely co-regulated with TGF-$\beta$. TGF-$\beta$ gene and protein expression during murine development has been surveyed extensively (Heine *et al.*, 1987; Lehnert and Akhurst, 1988; Pelton *et al.*, 1989; Pelton *et al.*, 1990a,b; Millan *et al.*, 1991); these studies have not

30 identified expression by skeletal muscle cells, chondrocytes, hepatocytes, ganglion cells, mucosal cells lining the gut, and epithelial cells of developing nephrons. It is conceivable that the LTBP-2 molecule has

an additional function in certain connective tissues
besides targeting TGF-β.

The binding properties of the LTBP-2 gene product
5    are under investigation.  Formally, the LTBP-2
polypeptide may bind a specific TGF-β isoform, another
member of the TGF-β superfamily (e.g., a bone
morphogenetic protein, inhibin, activin, or Mullerian
inhibiting factor), or a growth factor unrelated to TGF-
10   β.  Anti-peptide antibodies to the murine LTBP-2
polypeptide have been generated and osteoblast cell lines
that express the molecule at relatively high levels have
been identified.  Studies with these reagents suggest
that LTBP-2 assembles intracellularly into large latent
15   complexes with a growth factor that is being
characterized by immunological methods.

The presence of dibasic amino acids in the LTBP-2
sequence suggests that it may undergo cell- and tissue-
20   specific proteolysis.  TGF-β regulates extracellular
matrix production by suppressing matrix degradation
(through a decrease in the expression of proteases such
as collagenase, plasminogen activator, and stromelysin
plus an increase in the expression of proteinase
25   inhibitors such as plasminogen activator inhibitor-1 and
tissue inhibitor of metalloproteinase-1) and by
stimulating matrix macromolecule synthesis (for recent
review, see Lyons and Moses, 1990; Massague, 1990; Laiho
and Keski-Oja, 1992; Miyazono et al., 1992).  Conversely,
30   production of extracellular matrix has been shown to down
regulate TGF-β gene expression (Streuli et al., 1993).
TGF-β may therefore regulate extracellular matrix
production through a sophisticated feedback loop that
influences the expression of a relatively large number of
35   genes.  LTBP-1 and LTBP-2 may contribute to this
regulation by facilitating the assembly and secretion of
large latent growth factor complexes and then targeting

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the complex to specific connective tissues (Taipale
et al., 1994).

If LTBP-3 is like LTBP-1, it has the potential to
5    function as a secreted, extracellular structural protein.
As demonstrated here, domain #1 of LTBP-3 appears to be a
unique sequence that likely has a globular conformation.
Domain #1 also is highly basic and may facilitate LTBP-2
binding to acidic molecules (e.g., acidic proteoglycans)
10   within the extracellular space.  Sequences rich in basic
amino acids have also been shown to function as
endoproteolytic processing signals for several peptide
hormones (Barr, 1991; Steiner et al., 1992).  It is
possible, therefore, that the NH$_2$-terminus of LTBP-3 is
15   proteolytically processed in a tissue-specific manner.
Domains #2 and #4 consist of consecutive cysteine-rich
repeats, the majority of which are of the EGF-CB type.
Besides binding calcium (Corson et al., 1993), these
repeats may provide LTBP-3 with regions conformation
20   capable of interacting with other matrix macromolecules
(Engel, 1989).  Domain #3 is proline rich and may be
capable of bending (or functioning like a hinge) in
three-dimensional space (MacArthur and Thornton, 1991).
(In this regard, domain #2 is of interest because it has
25   a similar stretch of 135 amino acids that is both
proline- and glycine-rich.  Since glycine-rich sequences
are also thought to be capable of bending or functioning
like a hinge in three-dimensional space, this amino acid
sequence may interrupt the extended conformation of
30   domain #2, thereby providing it with a certain degree of
flexibility in three-dimensional space.)  Domain #5 also
appears to be a unique sequence having a globular
conformation.  The absence of a known cell attachment
motif may indicate that, in contrast to LTBP-1, the
35   LTBP-3 molecule may have a more limited role in the
extracellular matrix (i.e., that of a structural protein)

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in addition to its ability to target latent TGF-β
complexes to specific connective tissues.

5   MC3T3-E1 pre-osteoblasts co-express LTBP-3 and
TGF-β1 and these proteins form a complex in the culture
medium.  These results are particularly interesting
because bone represents one of the largest known
repositories of latent TGF-β (200 μg/kg bone; Seyedin
et al., 1986 and 1987), and because this growth factor
10  plays a critical role in the determination of bone
structure and function.  For example, TGF-β is thought to
(i) provide a powerful stimulus to bone formation in
developing tissues, (ii) function as a possible "coupling
factor" during bone remodeling (a process that
15  coordinates bone resorption and formation), and (iii)
exert a powerful bone inductive stimulus following
fracture.  Activation of the latent complex may be an
important step governing TGF-β effects, and LTBP may
modulate the activation process (e.g., it may "protect"
20  small latent complexes from proteolytic attack).

Expression of large latent TGF-β complexes bearing
LTBP may be physiologically relevant to, i.e., may be
part of the mechanism of, the pre-osteoblast → osteoblast
25  differentiation cascade.  This is based on the evidence
that MC3T3-E1 cells express large latent TGF-β1 complexes
bearing LTBP-2 precisely at the time of transition from
the pre-osteoblast to osteoblast phenotype (~day 14 in
culture, or, at the onset of alkaline phosphatase
30  expression; see Quarles et al., 1992).  The organ culture
model, for example, likely is comprised of differentiated
osteoblasts but few bond progenitors, making it a
difficult model at best in which to study the
differentiation cascade (Dallas et al., 1984).  It is
35  also well known that MG63, ROS17/2.8 and UMR 106 cells
are rapidly dividing and they express the osteoblast
phenotype.  Thus, these osteoblast-like cell lines do not

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show the uncoupling of cell proliferation and cell
differentiation that characterizes the normal
(physiologically relevant) pre-osteoblast → osteoblast
transition (Gerstenfeld et al., 1984; Stein and Lian,

5   1993).  Therefore, the production of small versus large
latent TGF-β complexes may be associated with specific
stages in the maturation of bone cells.


LTBP-3 may bind calcium, since EGF-CB repeats have

10  been shown to mediate high affinity calcium binding in
LTBP-1 and other proteins (Colosetti et al., 1993).
Calcium binding, in turn, may contribute to molecular
conformation and the regulation of its interactions with
other molecules.  The presence of dibasic amino acids

15  suggests that LTBP-3 may also undergo cell- and
tissue-specific proteolysis.  TGF-β regulates
extracellular matrix production by suppressing matrix
degradation (through a decrease in the expression of
proteases such as collagenase, plasminogen activator, and

20  stromelysin plus an increase in the expression of
proteinase inhibitors such as plasminogen activator
inhibitor-1 and tissue inhibitor of metalloproteinase-1)
and by stimulating matrix macromolecule synthesis (for
recent reviews, see Lyons and Moses, 1990; Massague,

25  1990; Laiho and Keski-Oja, 1992; and Miyanzono et al.,
1993).  Conversely, production of extracellular matrix
has been shown to down regulate TGF-β gene expression
(Streuli et al., 1993).  TGF-β may therefore regulate
extracellular matrix production through a sophisticated

30  feedback loop that influences the expression of a
relatively large number of genes.  LTBP-1, LTBP-2, and
LTBP-3 may contribute to this regulation by facilitating
the assembly and secretion of large latent growth factor

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complexes and then targeting the complex to specific
connective tissues (Taipale et al., 1994).

## EXAMPLE XVI

5    PREPARATION OF ANTIBODIES AGAINST THE LTBP-3 GENE PRODUCT


     An affinity-purified antibody (#274) capable of
immunoprecipitating was prepared against the murine LTBP-
3 gene product.  A full-length murine cDNA was assembled
10   into the pcDNA3 mammalian expression vector (Invitrogen)
and expressed following transient transfection of 293T
cells.  Nascent polypeptides, radiolabeled by addition of
$^{35}$S Cys to the medium of transfected cells, were
immunoprecipitated using affinity-purified antibody #274.
15   As shown in FIG. 20, the new murine polypeptide was
estimated to be 180-190 kDa.  To ensure the specificity
of #274 binding, we showed that preincubation with 10 $\mu$g
of synthetic peptide blocks immunoprecipitation of the
180-190 kDa band.
20

     Finally, MC3T3-E1 cells were cultured for 7 days
under differentiating conditions and double-labeled with
30 $\mu$Ci/ml $^{35}$S cysteine and $^{35}$S methionine in deficient
media.  Radiolabeled media was dialyzed into cold PBS
25   with protease inhibitors.  Aliquots of the dialyzed
medium sample ($10^6$ incorporated CPM) were analyzed by a
combined immunoprecipitation/Western analysis protocol.
The murine polypeptide was clearly and reproducibly
secreted by MC3T3-E1 cells, migrating under reducing
30   conditions as a single band of 180-190 kDa (FIG. 21).
Consistent with the results of previous studies (e.g.,
Miyazono et al., 1988; Dallas et al., 1994; Moren et al.,
1994), bands of 70 and 50 kDa corresponding to the TGF-β1
precursor were co-immunoprecipitated with the 180 kDa
35   LTBP-3 protein.  Weak bands of 40 and 12 kDa were also
identified in experiments in which only
immunoprecipitation was performed.  The latter were not

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included in FIG. 21 because they migrated within that
portion of the gel included in the Western analysis.
Protein bands of 70-12.5 kDa are not variant forms of
LTBP-3; FIG. 20 demonstrates that LTBP-3 migrates as a
5    single band of 180-190 kDa following transient
transfection of 293T cells, which fail to make TGF-$\beta$1.
By immunoprecipitation, a unique band consistent with
monomeric mature TGF-$\beta$1 was found in the LTBP-2
immunoprecipitate. Antibody #274 is incapable of binding
10   TGF-$\beta$1 as determined by radioimmunoassay using
commercially available reagents (R&D Systems) and the
manufacturer's suggested protocols. These results have
been reproduced in 6 independent experiments which
utilized 3 separate lots of MC3T3-E1 medium. Thus the
15   new murine LTBP-3 polypeptide binds TGF-$\beta$ *in vitro*.


## EXAMPLE XVII
### ISOLATION OF A GENE ENCODING MURINE LTBP-2

20       In addition to determining the DNA and corresponding
polypeptide sequence of the murine LTBP-3 gene, the
murine LTBP-2 gene was also cloned and sequenced.

      The complete cDNA nucleotide sequence for murine
25   LTBP-2 is shown in FIG. 27 (SEQ ID NO:17). The deduced
amino acid sequence is shown in FIG. 28 (SEQ ID NO:18).


## EXAMPLE XVIII
30   ### EXPRESSION OF RECOMBINANT TYPE II COLLAGEN

      The *Pichia* Expression Kit (Invitrogen, Inc.) may be
used to prepare recombinant type II collagen. This kit,
based on the methylotrophic yeast, *Pichia pastoris*,
35   allows high-level expression of recombinant protein in an
easy-to-use relatively inexpensive system. In the
absence of the preferred carbon source, glucose, *P*.

*pastoris* utilizes methanol as a carbon source. The *AOX1* promoter controls the gene that codes for the expression of the enzyme alcohol oxidase, which catalyzes the first step in the metabolism of methanol. This promoter, which

5 is induced by methanol, has been characterized and incorporated into a series of *Pichia* expression vectors. This feature of *Pichia* has been exploited to express high levels of recombinant proteins often in the range of grams per liter. Because it is eukaryotic, *P. pastoris*

10 utilizes posttranslational modification pathways that are similar to those used by mammalian cells. This implies that the recombinant type II collagen will be glycosylated and will contain disulfide bonds.

15      The inventors contemplate the following particular elements to be useful in the expression of recombinant type II collagen: the DNA sequence of human type II collagen (SEQ ID NO:11) (Lee et al., 1989); rat type II collagen (SEQ ID NO:13) (Michaelson, et al., 1994);

20 and/or mouse type II collagen (SEQ ID NO:15) (Ortman, et al., 1994). As other sources of DNA sequences encoding type II collagen are available, these three are examples of many sequence elements that may be useful in the present invention.

25

      For preparation of a recombinant type II collagen, the native type II collagen cDNA is modified by the addition of a commercially available epitope tag (the HA epitope, Pharmacia, LKB Biotechnology, Inc.). Such

30 fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102 (herein incorporated by reference) or by introducing selected

35 sequences into recombinant vectors for recombinant production. (PCR™ is a registered trademark of Hoffmann-LaRoche, Inc.). This is followed by cloning into the

*Pichia* expression vector. The resulting plasmid is characterized by DNA sequence analysis, linearized by digestion with *Not*I, and spheroplasts will be prepared and transformed with the linearized construct according
5    to the manufacturer's recommendations.

Transformation facilitates a recombination event *in vivo* between the 5' and 3' *AOX1* sequences in the *Pichia* vector and those in the *Pichia* genome. The result is the
10   replacement of *AOX1* with the gene of interest.

Transformants are then plated on histidine-deficient media, which will select for successfully transformed cells. Transformants are further selected against slow
15   growth on growth media containing methanol. Positive transformants are grown for 2 days in liquid culture and then for 2-6 days in broth that uses methanol as the sole carbon source. Protein expression is evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
20   PAGE) and Western hybridization using a commercially available polyclonal antisera to the HA epitope (Pharmacia).

Recombinant type II collagen protein can be purified
25   according to the manufacturer's recommendations, dialyzed against double distilled, deionized water and lyophilized in 10 mg aliquots. The aliquots are sterilized and used as implant material for the osteoconductive matrices.

30                            *       *       *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure.
35   While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations

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may be applied to the composition, methods and in the
steps or in the sequence of steps of the method described
herein without departing from the concept, spirit and
scope of the invention.  More specifically, it will be
5  apparent that certain agents which are both chemically
and physiologically related may be substituted for the
agents described herein while the same or similar results
would be achieved.  All such similar substitutes and
modifications apparent to those skilled in the art are
10 deemed to be within the spirit, scope and concept of the
invention as defined by the appended claims.

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      (ii) INVENTORS: BONADIO, Jeffrey
15                   ROESSLER, Blake J.
                   GOLDSTEIN, Steven A.
                   LIN, Wushan

    (iii) TITLE OF INVENTION:  METHODS AND COMPOSITIONS
20                            FOR STIMULATING BONE CELLS

     (iv) NUMBER OF SEQUENCES: 18

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          (A) MEDIUM TYPE: Floppy disk
          (B) COMPUTER: IBM PC compatible
35        (C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII
          (D) SOFTWARE: PatentIn Release #1.0, Version
                                #1.30

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        (vii) CURRENT APPLICATION DATA:
              (A) APPLICATION NUMBER:  UNKNOWN
              (B) FILING DATE: CONCURRENTLY HEREWITH
              (C) CLASSIFICATION: UNKNOWN

5

        (viii) PRIOR APPLICATION DATA:
              (A) APPLICATION NUMBER: US 08/316,650
              (B) FILING DATE: 30-SEP-1994
              (C) CLASSIFICATION: UNKNOWN

10

              (A) APPLICATION NUMBER: US 08/199,780
              (B) FILING DATE: 18-FEB-1994
              (C) CLASSIFICATION: UNKNOWN


15      (ix) ATTORNEY/AGENT INFORMATION:
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              (C) TELEX: 79-0924


25

     (2) INFORMATION FOR SEQ ID NO:1:


        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 417 amino acids
30            (B) TYPE: amino acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear


        (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ile Pro Gly Asn Arg Met Leu Met Val Val Leu Leu Cys Gln Val
1               5                   10                  15

Leu Leu Gly Gly Ala Thr Asp Ala Ser Leu Met Pro Glu Thr Gly Lys
            20                  25                  30

Lys Lys Val Ala Glu Ile Gln Gly His Ala Gly Gly Arg Arg Ser Gly
        35                  40                  45

Gln Ser His Glu Leu Leu Arg Asp Phe Glu Ala Thr Leu Leu Gln Met
    50                  55                  60

Phe Gly Leu Arg Arg Pro Gln Pro Ser Lys Ser Ala Val Ile Pro
65                  70                  75                  80

Asp Tyr Met Ser Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu Glu
            85                  90                  95

Glu Glu Gln Ser Gln Gly Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala
        100                 105                 110

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Ser Ser Ala Asn Thr Val Ser Ser Phe His His Glu Glu His Leu Glu
                115                 120                 125

Asn Ile Pro Gly Thr Ser Glu Ser Ser Ala Phe Arg Phe Phe Asn
            130                 135                 140

Leu Ser Ser Ile Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg
                145                 150                 155                 160

Leu Phe Arg Glu Gln Val Asp Gln Gly Pro Asp Trp Glu Gln Gly Phe
                165                 170                 175

His Arg Met Asn Ile Tyr Glu Val Met Lys Pro Pro Ala Glu Met Val
                180                 185                 190

Pro Gly His Leu Ile Thr Arg Leu Leu Asp Thr Ser Leu Val Arg His
                195                 200                 205

Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro Ala Val Leu Arg
                210                 215                 220

Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu Val Thr
                225                 230                 235                 240
```

His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Ser Ile Ser
245                 250                 255

Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu
260                 265                 270

Leu Val Thr Phe Gly His Asp Gly Arg Gly His Thr Leu Thr Arg Arg
275                 280                 285

Ser Ala Lys Arg Ser Pro Lys His His Pro Gln Arg Ser Ser Lys Lys
290                 295                 300

Asn Lys Asn Cys Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val
305                 310                 315                 320

Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr
325                 330                 335

Cys His Gly Asp Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr
340                 345                 350

Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile
355                 360                 365

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Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu
370                     375                     380

Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met
385                 390                 395                 400

Val Val Glu Gly Cys Gly Cys Arg Tyr Pro Tyr Asp Val Pro Asp Tyr
405                 410                 415

Ala

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 3753 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

    (A) NAME/KEY: CDS

    (B) LOCATION: 1..3753

5    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG    48
Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Leu Ala Leu
  1               5                  10                  15
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```
CTG GGC CCC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG        96
Leu Gly Pro Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln
             20                  25                  30
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```
GCG GGG GCG GGG CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT   144
Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro
         35                  40                  45
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```
GTG ATC TGC AAG CGG ACC TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT   192
Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys
     50                  55                  60
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```
     CAG CAG GGC TCC AAC ATG ACG CTC ATC GGA GAG AAC GGC CAC AGC ACC    240
     Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr
         65                  70                  75                  80

 5   GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG GTG TGC CCT CTA CCC        288
     Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Cys Pro Leu Pro
             85                  90                  95

10   TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG TGC CTG TGT CCC    336
     Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro
                100                 105                 110

     CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA ACC GGA    384
     Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly
             115                 120                 125

15   GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC    432
     Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser
                130                 135                 140

     ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC    480
     Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser
             145                 150                 155                 160
20
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                                                                    528
AAA CAC GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG
Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro
                165                     170                 175

                                                                    576
  5  GGG GAG GGT CCT CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG
     Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly
                     180                     185                 190

                                                                    624
 10  CCA GGA CAA ATC TCG GCA GAA GTG CAG GCT CCG CCC GTG GTG AAC
     Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Val Val Asn
                     195                     200             205

                                                                    672
 15  GTG CGT GTC CAT CAC CCT CCT GAA GCT TCC GTT CAG GTG CAC CGC ATC
     Val Arg Val His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile
                     210                     215                 220

                                                                    720
     GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT TCC CAG CAC TTG CTG CCG
     Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro
                     225                     230             235     240

                                                                    768
 20  CAT CCC AAG CCC CCG CAC CCG AGG CCA CCC ACT CAA AAG CCA CTG GGC
     His Pro Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly
                     245                     250             255
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```
CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC AAC CCT    816
Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro
            260                 265                 270

TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT    864
Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr
            275                 280                 285

GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA    912
Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr
            290                 295                 300

GGG GTG CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC    960
Gly Val Gln Lys Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys
        305                 310                 315             320

CCC CAG GGC TAC AAG AGG CTC AAC AGC ACC CAC TGC CAG GAT ATC AAC    1008
Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr His Cys Gln Asp Ile Asn
            325                 330                 335

GAA TGT GCG ATG CCC GGG AAT GTG TGC CAT GGT GAC TGC CTC AAC AAC    1056
Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn
            340                 345                 350
```

CCT GGC TCT TAT CGC TGT GTC TGC CCG CCC GGT CAT AGC TTG GGT CCC    1104
Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro
355                             360                         365

CTC GCA GCA CAG TGC ATT GCC GAC AAA CCA GAG GAG AAG AGC CTG TGT    1152
Leu Ala Ala Gln Cys Ile Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys
370                             375                         380

TTC CGC CTT GTG AGC ACC GAA CAC CAG TGC CAG CAC CCT CTG ACC ACA    1200
Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His Pro Leu Thr Thr
385                             390                         400

CGC CTA ACC CGC CAG CTC TGC TGC TGT AGT GTG GGT AAA GCC TGG GGT    1248
Arg Leu Thr Arg Gln Leu Cys Cys Cys Ser Val Gly Lys Ala Trp Gly
405                             410                         415

GCC CGG TGC CAG CGC TGC CCG GCA GAT GGT ACA GCA GCC TTC AAG GAG    1296
Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu
420                             425                         430

ATC TGC CCC GGC TGG GAA AGG GTA CCA TAT CCT CAC CTC CCA CCA GAC    1344
Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp
435                             440                         445

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GCT CAC CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC    1392
Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Leu Pro Ala Pro Asp
     450             455                 460

GGG CCA CCC AAA CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCA    1440
Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro
465                 470                 475             480

CCC CTC GAG GAC ACA GAG GAA GAG AGA GGA GTG ACC ATG GAT CCA CCA    1488
Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro
                485                 490                 495

GTG AGT GAG GAG CGA TCG GTG CAG CAG AGC CAC CCC ACT ACC ACC ACC    1536
Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr Thr
                500                 505                 510

TCA CCC CCC CGG CCT TAC CCA GAG CTC ATC TCT CGC CCC TCC CCA CCT    1584
Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro
                515                 520                 525

ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC CGA AGT GCA GTG    1632
Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val
                530                 535                 540
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```
      GAG ATC GCC CCC ACT CAG GTC ACA GAG ACC GAT GAG TGC CGA TTG AAC    1680
      Glu Ile Ala Pro Thr Gln Val Thr Glu Thr Asp Glu Cys Arg Leu Asn
      545                   550                   555                   560

5     CAG AAT ATC TGT GGC CAT GGA CAG TGT GTG CCT GGC CCC TCG GAT TAC    1728
      Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr
                    565                   570                   575

10    TCC TGC CAC TGC AAC GCT GGC TAC CGG TCA CAC CCG CAG CAC CGC TAC    1776
      Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr
                    580                   585                   590

      TGT GTT GAT GTG AAC GAG TGC GAG GCA GAG CCC TGC GGC CCC GGG AAA    1824
      Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys
                    595                   600                   605

15    GGC ATC TGT ATG AAC ACT GGT GGC TCC TAC AAT TGT CAC TGC AAC CGA    1872
      Gly Ile Cys Met Asn Thr Gly Gly Ser Tyr Asn Cys His Cys Asn Arg
                    610                   615                   620

20    GGC TAC CGC CTC CAC GTG GGT GCA GGG GGC CGC TCG TGC GTG GAC CTG    1920
      Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val Asp Leu
                    625                   630                   635                   640
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```
5    AAC GAG TGC GCC AAG CCT CAC CTG TGT GGG GAC GGT GGC TTC TGC ATC    1968
     Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile
                     645                         650              655

     AAC TTC CCT GGT CAC TAC AAA TGC AAC TGC TAT CCT GGC TAC CGG CTC    2016
     Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu
                         660                         665          670

10   AAG GCC TCC CGA CCG CCC ATT TGC GAA GAC ATC GAC GAG TGT CGC GAC    2064
     Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp
             675                         680              685

     CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA CCT GGC AGC TTC    2112
     Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe
             690                         695                   700

15   AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC    2160
     Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Gly Ala
             705                         710              715     720

     TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA    2208
20   Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly
             725                         730              735
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```
      TGG TGT GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG    2256
      Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly
                      740                     745                     750

 5    ATA CGA ACC CGC ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT    2304
      Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys
                      755                     760                     765

10    GAG GCT GGG AAA GTG TGC CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC    2352
      Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly
                      770                     775                     780

      TCT TTC CAG TGT CAG TGC CTC TCC GGC TAT CAT CTG TCA AGG GAT CGG    2400
      Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg
15                    785                     790                     795                     800

      AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC TTC CCT GCG GCC TGC ATC    2448
      Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile
                      805                     810                     815

20    GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA TGT CTC TGT CCC    2496
      Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro
                      820                     825                     830
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```
    CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT ATA GAT    2544
    Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp
                        840                            845

5   GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC    2592
    Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn
            850                        855                    860

10  CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC    2640
    Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Leu Thr Thr
        865                    870                    875            880

15  CAG GAC CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG    2688
    Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys
                    885                    890                    895

    GAG TGC TAC CTT AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG    2736
    Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu
                900                    905                    910

20  GCT ACC AAT GTC ACT CAG CAG GAA TGC TGT CTG TGC TCT CTG GGA GCT GGC    2784
    Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Leu Cys Ser Leu Gly Ala Gly
            915                    920                    925
```

```
     TGG GGA GAC CAC TGC GAA ATC TAT CCC TGT CCA GTC TAC AGC TCA GCC    2832
     Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala
                             930                     935             940

5    GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA AGG CTA CAC TCA GGA CAA    2880
     Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln
                     945                 950                 955     960

10   CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC ATC GAC GAA TGC    2928
     Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys
                 965                 970                     975

15   ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG AAC TCG    2976
     Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser
             980                     985                     990

     CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC GAT GGC        3024
     Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Asp Gly
         995                 1000                1005

20   AAC CTG CTG GAG TGC GTG GAC GAG TGC TTG GAT GAG TCT AAC            3072
     Asn Leu Leu Glu Cys Val Asp Glu Cys Leu Asp Glu Ser Asn
         1010                1015                1020
```

```
      TGC AGG AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC      3120
      Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys
      1025                     1030                     1035            1040

 5    ACT CCG CCG GCA GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG ATC CCG      3168
      Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro
                1045                     1050                     1055

10    GAG AGA TGG AGC ACG CCC CAG AGA GAC GTG AAG TGT GCT GGG GCC AGC      3216
      Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser
                     1060                     1065                     1070

      GAG GAG AGG ACG GCA TGT GTA TGG GGC CCC TGG GCG GGA CCT GCC CTC      3264
      Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu
15              1075                     1080                     1085

      ACT TTT GAT GAC TGC TGC CGC CAG CCG CGG CTG GGT ACC CAG TGC          3312
      Thr Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys
                     1090                     1095                     1100

20    AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC CCG ACT TCA CAG      3360
      Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln
      1105                     1110                     1115            1120
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AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG GGG AAG  3408
Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys
        1125             1130            1135

5  TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT  3456
Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg
        1140             1145            1150

10 TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG  3504
Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu
        1155             1160            1165

TGT CCT GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC  3552
Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp
15   1170            1175            1180

ATT GAT GAG TGC CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC  3600
Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser
 1185           1190           1195           1200

20 GAG CGG TGC GTG AAC ACC AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT  3648
Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala
        1205             1210            1215

```
GGC TTC ACG CGC AGC CGC CCT CAC GGG CCT GCG TGC CTC AGC GCC GCC    3696
Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala
                    1220                1225                1230

GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA GTG ATC GAT CAT CGA GGG    3744
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly
                1235                1240                1245

TAT TTT CAC    3753
Tyr Phe His
        1250
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1251 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Gln Ala Ala Leu Gly Leu Leu Leu Ala Leu Leu Leu Ala Leu
1               5                   10                  15

Leu Gly Pro Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln
            20                  25                  30

Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro
        35                  40                  45

Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys
    50                  55                  60

Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr
65                  70                  75                  80

Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Val Cys Pro Leu Pro
            85                  90                  95

Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro
        100                 105                 110

Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly
    115                 120                 125

5

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Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser
130                                     140

5   Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser
145                 150                 155                 160

Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro
165                 170                 175

10  Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly
180                 185                 190

Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Val Val Asn
195                 200                 205

15  Val Arg Val His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile
210                 215                 220

Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro
20  225                 230                 235                 240

His Pro Lys Pro Pro His Pro Arg Pro Thr Gln Lys Pro Leu Gly
245                 250                 255

Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro
260                 265                 270

5  Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr
      275                 280                 285

Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr
290                 295                 300

10  Gly Val Gln Lys Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys
       305                 310                 315                 320

Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr His Cys Gln Asp Ile Asn
325                 330                 335

15  Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn
       340                 345                 350

Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro
355                 360                 365

20  Leu Ala Ala Gln Cys Ile Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys
       370                 375                 380

```
      Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His Pro Leu Thr Thr
      385                                     395                 400

5     Arg Leu Thr Arg Gln Leu Cys Cys Cys Ser Val Gly Lys Ala Trp Gly
                             405                             415

      Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu
                                 420                             430

10    Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp
      435                                  440                     445

      Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp
      450                                  455                     460

15    Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro
      465                                  470                     480

      Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro
                                 485                             495

20    Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr
      500                                  505                     510
```

```
      Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro
      515                     520                     525

      Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val
5     530                     535                     540

      Glu Ile Ala Pro Thr Gln Val Thr Glu Thr Asp Glu Cys Arg Leu Asn
10    545                     550                     555                 560

      Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr
      565                     570                     575

      Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr
15    580                     585                     590

      Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys
      595                     600                     605

      Gly Ile Cys Met Asn Thr Gly Gly Ser Tyr Asn Cys His Cys Asn Arg
20    610                     615                     620

      Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val Asp Leu
      625                     630                     635                 640
```

```
    Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile
                        645                 650                 655

    Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu
                        660                 665                 670

    Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp
                        675                 680                 685

10  Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe
                        690                 695                 700

15  Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Gly Ala
        705                 710                 715                 720

    Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly
                        725                 730                 735

20  Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly
                        740                 745                 750

    Ile Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys
                        755                 760                 765
```

```
    Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly
                770                 775                 780

5   Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg
                785                 790                 795             800

    Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile
                805                 810                 815

10  Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro
                820                 825                 830

    Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp
                835                 840                 845

15  Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn
                850                 855                 860

    Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr
                865                 870                 875             880

20  Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys
                885                 890                 895
```

```
      Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu
                           900                     905                     910

      Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser Leu Gly Ala Gly
5                          915                     920                     925

      Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala
                           930                     935                     940

10    Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln
                           945                     950                     955                     960

      Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys
                           965                     970                     975

15    Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser
                           980                     985                     990

20    Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly
                           995                     1000                    1005

      Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn
                           1010                    1015                    1020
```

Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys
1025                1030                1035                1040

Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro
                1045                1050                1055

5

Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser
                1060                1065                1070

10

Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu
                1075                1080                1085

Thr Phe Asp Asp Cys Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys
                1090                1095                1100

15

Arg Pro ... Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln
1105                1110                1115                1120

Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys
                1125                1130                1135

20

Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg
                1140                1145                1150

Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu
1155                    1160                    1165

5    Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp
1170                    1175                    1180

Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser
1185                    1190                    1195                    1200

10   Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala
1205                    1210                    1215

Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala
1220                    1225                    1230

15   Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly
1235                    1240                    1245

Tyr Phe His
20   1250

(2)  INFORMATION FOR SEQ ID NO:4:

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        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
5           (D) TOPOLOGY: linear


    (ii) MOLECULE TYPE: DNA (genomic)


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
10
    AACATGACGC TCATCGGAGA GAAC                                24


    (2) INFORMATION FOR SEQ ID NO:5:


15      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 18 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
20
    (ii) MOLECULE TYPE: DNA (genomic)


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:


25  AGGTGATCGC AGATCCTC                                       18



    (2) INFORMATION FOR SEQ ID NO:6:


30      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
35
    (ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TACCGATGCT ACCGCAGCAA TCTT                                    24

5


(2) INFORMATION FOR SEQ ID NO:7:


    (i) SEQUENCE CHARACTERISTICS:

10        (A) LENGTH: 22 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear


15    (ii) MOLECULE TYPE: DNA (genomic)


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGCCTAAAC TCTACCAGCA CG                                      22

20


(2) INFORMATION FOR SEQ ID NO:8:


    (i) SEQUENCE CHARACTERISTICS:

25        (A) LENGTH: 22 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear


30    (ii) MOLECULE TYPE: DNA (genomic)


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGTCACGTC ATCCATTCCA CA                                      22

35

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(2) INFORMATION FOR SEQ ID NO:9:


    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
5       (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear


    (ii) MOLECULE TYPE: DNA (genomic)
10

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:


CGTCCAAGTT GTGTCTTAGC AG                                    22


15

(2) INFORMATION FOR SEQ ID NO:10:


    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 53 amino acids
20      (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear


    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Pro Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro Lys Gly
1               5                   10                  15

Gln Thr Gly Glu Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln Gly Pro
            20                  25                  30

Lys Gly Glu Thr Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly Pro Ala
        35                  40                  45

Gly Glu Glu Gly Lys
        50

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 159 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCCTCCCG GTCCTCAAGG TGCAACTGGT CCTCTGGGCC CCAAAGGTCA GACGGGTGAG    60

CCCGGCATCG CTGGCTTCAA AGGTGAACAA GGCCCCAAGG GAGAGACTGG ACCTGCTGGG   120

CCCCAGGGAG CCCCTGGCCC TGCTGGTGAA GAAGGAAAA                          159

(2) INFORMATION FOR SEQ ID NO:12:

　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　(A) LENGTH: 1442 amino acids
　　　　　(B) TYPE: amino acid
　　　　　(C) STRANDEDNESS: single
　　　　　(D) TOPOLOGY: linear

　　　(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ile Arg Leu Gly Ala Pro Gln Ser Leu Leu Val Leu Leu Thr Leu Leu
1     5     10     15

Ile Ala Ala Val Leu Arg Cys Gln Gly Gln Asp Ala Gln Glu Ala Gly
20     25     30

Ser Cys Leu Gln Asn Gly Gln Arg Tyr Lys Asp Lys Asp Val Trp Lys
35     40     45

Pro Ser Ser Cys Arg Ile Cys Val Cys Asp Thr Gly Asn Val Leu Cys
50     55     60

Asp Asp Ile Ile Cys Glu Asp Pro Asp Cys Leu Asn Pro Glu Ile Pro
65     70     75     80

Phe Gly Glu Cys Cys Pro Ile Cys Pro Ala Asp Leu Ala Thr Ala Ser
85     90     95

Gly Arg Lys Leu Gly Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile
100     105     110

Arg Asp Gly Pro Ala Gly Glu Gln Gly Pro Arg Gly Asp Arg Gly Asp
115     120     125

5

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Lys Gly Glu Lys Asn Phe Ala Ala Gln Met Ala Gly Gly Tyr Asp Glu
130                             135                             140

5        Lys Ala Gly Gly Ala Gln Met Gly Val Met Gln Gly Pro Met Gly Pro
         145                             150                             155                    160

         Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Ala Pro Gly Pro Gln
                                  165                             170                             175

10       Gly Phe Gln Gly Asn Pro Gly Glu Pro Gly Glu Pro Gly Val Ser Gly
                  180                             185                             190

         Pro Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Lys Pro Gly Asp
                  195                             200                             205

15       Asp Gly Glu Ala Gly Lys Pro Gly Lys Ser Gly Glu Arg Gly Leu Pro
         210                             215                             220

         Gly Pro Met Gly Ala Arg Gly Phe Pro Gly Thr Pro Gly Leu Pro Gly
         225                             230                             235                    240

20       Val Lys Gly His Arg Gly Tyr Pro Gly Leu Asp Gly Ala Lys Gly Glu
                  245                             250                             255

Ala Gly Ala Pro Gly Val Lys Gly Glu Ser Gly Ser Pro Gly Glu Asn
                    260                 265                 270

Gly Ser Pro Gly Pro Met Gly Pro Arg Gly Leu Pro Gly Glu Arg Gly
            275                 280                 285

Arg Thr Gly Pro Ala Gly Ala Ala Arg Gly Asn Asp Gly Gln
        290                 295                 300

Pro Gly Pro Ala Gly Pro Pro Gly Pro Val Gly Pro Ala Gly Gly Pro
    305                 310                 315                 320

Gly Phe Pro Gly Ala Pro Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly
                325                 330                 335

Ala Arg Gly Pro Glu Gly Ala Gln Gly Ser Arg Gly Glu Pro Gly Asn
                340                 345                 350

Pro Gly Ser Pro Gly Pro Ala Gly Ala Ser Gly Asn Pro Gly Thr Asp
            355                 360                 365

Gly Ile Pro Gly Ala Lys Gly Ser Ala Gly Ala Pro Gly Ile Ala Gly
        370                 375                 380

Ala Pro Gly Phe Pro Gly Pro Arg Gly Pro Pro Gly Pro Gln Gly Ala
385                390                395                400

Thr Gly Pro Leu Gly Pro Lys Gly Gln Ala Gly Glu Pro Gly Ile Ala
                405                410                415

Gly Phe Lys Gly Asp Gln Gly Pro Lys Gly Glu Thr Gly Pro Ala Gly
                420                425                430

Pro Gln Gly Ala Pro Gly Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala
                435                440                445

Arg Gly Glu Pro Gly Gly Gly Ala Gly Pro Ile Gly Pro Pro Gly Glu Arg
                450                455                460

Gly Ala Pro Gly Asn Arg Gly Phe Pro Gly Gln Asp Gly Leu Ala Gly
465                470                475                480

Pro Lys Gly Ala Pro Gly Glu Arg Gly Pro Ser Gly Leu Ala Gly Pro
                485                490                495

Lys Gly Ala Asn Gly Asp Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro
                500                505                510

Gly Ala Arg Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly
515                     520                     525

Lys Val Gly Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro
530                     535                     540

Pro Gly Pro Gln Gly Ala Arg Gly Gln Pro Gly Val Met Gly Phe Pro
545                     550                     555                     560

Gly Pro Lys Gly Ala Asn Gly Glu Pro Gly Lys Ala Gly Glu Lys Gly
565                     570                     575

Leu Ala Gly Ala Pro Gly Leu Arg Gly Leu Pro Gly Lys Asp Gly Glu
580                     585                     590

Thr Gly Ala Ala Gly Pro Pro Gly Pro Ser Gly Pro Ala Gly Glu Arg
595                     600                     605

Gly Glu Gln Gly Ala Pro Gly Pro Ser Gly Phe Gln Gly Leu Pro Gly
610                     615                     620

Pro Pro Gly Pro Pro Gly Glu Gly Gly Lys Gln Gly Asp Gln Gly Ile
625                     630                     635                     640

5

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15

20

Pro Gly Glu Ala Gly Ala Pro Gly Leu Val Gly Pro Arg Gly Glu Arg
                          645                 650                 655

Gly Phe Pro Gly Glu Arg Gly Ser Pro Gly Ala Gln Gly Leu Gln Gly
5                         660                 665                 670

Pro Arg Gly Leu Pro Gly Thr Pro Gly Thr Asp Gly Pro Lys Gly Ala
                          675                 680                 685

Ala Gly Pro Asp Gly Pro Pro Gly Ala Gln Gly Pro Pro Gly Leu Gln
10                        690                 695                 700

Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Ile Ala Gly Pro Lys Gly
                          705                 710                 715         720

Asp Arg Gly Asp Val Gly Glu Lys Gly Pro Glu Gly Ala Pro Gly Lys
15                        725                 730                 735

Asp Gly Gly Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala
                          740                 745                 750

Gly Ala Asn Gly Glu Lys Gly Glu Ala Gly Pro Pro Gly Pro Ser Gly
20                        755                 760                 765

```
Ser Thr Gly Ala Arg Gly Ala Pro Gly Glu Pro Gly Glu Thr Gly Pro
                       775                     780

Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln Pro
785                     790                     795                 800

Gly Ala Lys Gly Asp Gln Gly Glu Ala Gly Gln Lys Gly Asp Ala Gly
                       805                     810             815

Ala Pro Gly Pro Gln Gly Pro Ser Gly Ala Pro Gly Pro Gln Gly Pro
                       820                     825                 830

Thr Gly Val Thr Gly Pro Lys Gly Ala Arg Gly Ala Gln Gly Pro Pro
                       835                     840             845

Gly Ala Thr Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly
                       850                     855                 860

Ala Asn Gly Asn Pro Gly Pro Ala Gly Pro Pro Gly Pro Ala Gly Lys
865                     870                     875                 880

Asp Gly Pro Lys Gly Val Arg Gly Asp Ser Gly Pro Pro Gly Arg Ala
                       885                     890             895
```

```
Gly Asp Pro Gly Leu Glu Gly Pro Ala Gly Glu Lys Gly
                                            905                 910

  5   Glu Pro Gly Asp Asp Gly Pro Ser Gly Leu Asp Gly Pro Pro Gly Pro
                          915                 920                 925

      Gln Gly Leu Ala Gly Gln Arg Gly Ile Val Gly Leu Pro Gly Gln Arg
                      930                 935                 940

 10   Gly Glu Arg Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly
                  945                 950                 955                 960

      Lys Gln Gly Ala Pro Gly Ala Ser Gly Asp Arg Gly Pro Pro Gly Pro
                      965                 970                 975

 15   Val Gly Pro Pro Gly Leu Thr Gly Pro Ala Gly Glu Pro Gly Arg Glu
                  980                 985                 990

      Gly Ser Pro Gly Ala Asp Gly Pro Pro Gly Arg Asp Gly Ala Ala Gly
                      995                1000                1005

 20   Val Lys Gly Asp Arg Gly Glu Thr Gly Ala Leu Gly Ala Pro Gly Ala
                  1010                1015                1020
```

```
Pro Gly Pro Pro Gly Ser Pro Gly Pro Ala Gly Pro Thr Gly Lys Gln
        1025            1030            1035            1040

    Gly Asp Arg Gly Glu Ala Gly Ala Gln Gly Pro Met Gly Pro Ser Gly
 5          1045            1050            1055

    Pro Ala Gly Ala Arg Gly Ile Ala Gly Pro Gln Gly Pro Arg Gly Asp
10          1060            1065            1070

    Lys Gly Glu Ser Gly Glu Gln Gly Glu Arg Gly Leu Lys Gly His Arg
            1075            1080            1085

    Gly Phe Thr Gly Leu Gln Gly Leu Pro Gly Pro Pro Gly Pro Ser Gly
15          1090            1095            1100

    Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly Pro Arg Gly Pro
            1105            1110            1115            1120

    Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ser Asn Gly Ile Pro
20          1125            1130            1135

    Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser Gly Glu Thr Gly
            1140            1145            1150
```

Pro Val Gly Pro Pro Gly Ser Pro Gly Pro Pro Gly Pro
1155                1160                1165

Pro Gly Pro Gly Ile Asp Met Ser Ala Phe Ala Gly Leu Gly Gln Arg
1170                1175                1180

Glu Lys Gly Gly Pro Asp Pro Met Gln Tyr Met Arg Ala Asp Glu Ala Asp
1185                1190                1195                1200

Ser Thr Leu Arg Gln His Asp Val Glu Val Asp Ala Thr Leu Lys Ser
1205                1210                1215

Leu Asn Asn Gln Ile Glu Ser Ile Arg Ser Pro Asp Gly Ser Arg Lys
1220                1225                1230

Asn Pro Ala Arg Thr Cys Gln Asp Leu Lys Leu Cys His Pro Glu Trp
1235                1240                1245

Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly Cys Thr Leu Asp
1250                1255                1260

Ala Met Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr Cys Val Tyr
1265                1270                1275                1280

5

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```
Pro Asn Pro Ala Thr Val Pro Arg Lys Asn Trp Trp Ser Ser Lys Ser
                        1285              1290              1295

Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Met Asn Gly Gly Phe
                        1300              1305              1310

His Phe Ser Tyr Gly Asp Gly Asn Leu Ala Pro Asn Thr Ala Asn Val
                        1315              1320              1325

Gln Met Thr Phe Leu Arg Leu Leu Ser Thr Glu Gly Ser Gln Asn Ile
                        1330              1335              1340

Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Leu Asp Glu Ala Ala Gly
                        1345              1350         1355         1360

Asn Leu Lys Lys Ala Leu Leu Ile Gln Gly Ser Asn Asp Val Glu Met
                        1365              1370              1375

Arg Ala Glu Gly Asn Ser Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly
                        1380              1385              1390

Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg
                        1395              1400              1405
```

5

10

15

20

Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro Met Asp
        1410              1415                 1420

Ile Gly Gly Ala Glu Gln Glu Phe Gly Val Asp Ile Gly Pro Val Cys
1425              1430                 1435                 1440

Phe Leu

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 267 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATAGGCCCTT TGGAGACGGC TGTTTTCCAG ACTCCAAACT ATCGTGTCAC ACGTGTGGGA 60

AATGAAGTGT CTTTCAATTG TGAGCAAACC CTGGACCACA ATACTATGTA CTGGTACAAG 120

CAAGACTCTA AGAAATTGCT GAAGATTATG TTTAGCTACA ATAATAAGCA ACTCATTGTA 180

AACGAAAACAG TTCCAAGGCG CTTCTCACCT CAGTCTTCAG ATAAAGCTCA TTTGAATCTT 240

CGAATCAAGT CTGTAGAGCT GGAGGAC 267

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Gly Pro Ser Gly Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly
1               5                   10                  15

Pro Arg Gly Pro Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ala
            20                  25                  30

Asn Gly Ile Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser
            35                  40                  45

Gly Glu Thr Gly Pro Ala
            50
```

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 731 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
AGAATATAGA TAGATATGTC TGTGCTGACC GTGGCCTTTT GCCTCTTCCT TCTACACAGG      60

GTCCCTTCTGG AGACCAAGGT GCTTCTGGTC CTGCTGGTCC TTCTGGCCCT AGAGTAAGTG     120

ACATGGGAGTT GGAAGATGGA GGGGGCCCTT CAGAGAGTGT GGGCCTGTGT TCCCATGGGG     180

AGGGAAATGC TGCTGCTTCT GGGGAAGCTG TGGGCTCAGG GGTCCTCACT CAGTAATGGG     240

GGCAGGACTG GCTCATGTGC CTATGGCCAG AAAAGCGCCT GAGGCCACAA TGGCTGTAAG     300

ACAAACATGA ATCAGCCTCT CGCTGTCAGA CAGAACAGCA TTTTACAAAG AGGAGCTTAG     360

GAGGGTAGGC AAGCCATGGA GCTATCCTGC TGGTTCTTGG CCAAATAGAG ACCAACTTAG     420

GGTTCCATGA CTGAGCATGT GAAGAACTGG GGGCGGAGTG GCTGGTGCTA TCAGGACAGC     480

CACCTACCCA GCCCCAGCGA CTCCCCAGCC TTCCCCTGTGG TGACCACTCT TTCCTCACGA     540

CCTCTCTCTC TTGCAGGGTC CTCCTGGCCC CGTCGGTCCC TCTGGCAAAG ATGGTGCTAA     600

TGGAATCCCT GGCCCCATTG GGCCTCCTGG TCCCCGTGGA CGATCAGGCG AAACCGGCCC     660
```

TGCTGTAAGT GTCCTGACTC CTTCCCTGCT GTCGAGGTGT CCCTACCATC CGGGAGGCTT          720

GAGCTCTTTT T                                                              731

(2) INFORMATION FOR SEQ ID NO:16:

       (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 14 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Cys
1               5                   10

(2) INFORMATION FOR SEQ ID NO:17:

-213-

(1)  SEQUENCE CHARACTERISTICS:

     (A) LENGTH: 5502 base pairs

     (B) TYPE: nucleic acid

     (C) STRANDEDNESS: single

     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

     (A) DESCRIPTION: /desc = "DNA"

(ix) FEATURE:

     (A) NAME/KEY: CDS

     (B) LOCATION: 1..5502

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG GAG AGC ACC TCC CCG CGA GGT CTC CGG TGC CCA CAG CTC TGC AGC      48
Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser
         1255               1260               1265

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CAC TCT GGC GCC ATG AGA GCG CCG ACC ACC GCT CGC TGC TCC GGA TGC    96
His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys
        1270            1275            1280

ATC CAA CGG GTG CGT TGG AGG GGC TTC CTG CCA CTT GTC CTG GCT GTC    144
Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val
        1285            1290            1295

TTG ATG GGG ACA AGT CAT GCC CAA CGG GAT TCC ATA GGG AGA TAC GAA    192
Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu
        1300            1305            1310            1315

CCA GCT AGC AGG GAT GCG AAT CGG TTG TGG CAC CCC GTG GGC AGC CAC    240
Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His
        1320            1325            1330

CCC GCA GCG GCT GCA GCC AAG GTG TAC AGT CTG TTC CGA GAG CCT GAC    288
Pro Ala Ala Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp
        1335            1340            1345

GCG CCG GTC CCC GGC TTG TCG CCC TCT GAG TGG AAC CAG GCC CAG    336
Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln
        1350            1355            1360
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      GGG AAC CCG GGA TGG CTC GCA GAG GCC GAG GCC AGG AGG CCA CCT CGA   384
      Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg
      1365              1370                  1375

 5    ACC CAG CAG CTG CGT CGA GTC CAG CCA CCT GTC CAG ACT CGG AGA AGC   432
      Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser
      1380              1385                  1390              1395

10    CAT CCC CGG GGC CAG CAG CAG ATA GCA GCC CGG GCT GCA CCT TCT GTC   480
      His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Pro Ser Val
      1400              1405                  1410

15    GCG CGC CTG GAA ACC CCT CAG CGA CCC GCG GCT GCA CGG CGA GGG CGG   528
      Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Arg Arg Gly Arg
      1415              1420                  1425

      CTC ACT GGG AGA AAT GTC TGC GGG GGA CAG TGC TGC CCA GGA TGG ACA   576
      Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr
      1430              1435                  1440

20    ACA TCA AAC AGC ACC AAC CAC TGT ATC AAA CCT GTG TGT CAG CCT CCC   624
      Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro
      1445              1450                  1455
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      TGT CAG AAC CGA GGC TCC TGC AGC AGG CCC CAG GTC TGC ATC TGC CGT    672
      Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg
      1460              1465                  1470                  1475

 5    TCT GGC TTC CGT GGG GCG CGC TGT GAG GAG GTC ATC CCT GAG GAG GAA    720
      Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu
      1480              1485                  1490

10    TTT GAC CCT CAG AAT GCC AGG CCT GTG CCC AGA CGC TCA GTG GAG AGA    768
      Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg
      1495              1500                  1505

      GCA CCC GGT CCT CAC AGA AGC AGT GAG GCC AGA GGA AGT CTA GTG ACC    816
      Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr
15    1510              1515                  1520

      AGA ATA CAG CCG CTG GTA CCA CCA TCA CCT CCT CGG CGC                864
      Arg Ile Gln Pro Leu Val Pro Pro Ser Pro Pro Arg Arg
      1525              1530                  1535

20    CTC AGC CAG CCC TGG CCC CTG CAG CAG CAC TCA GGG CCG TCC AGG ACA    912
      Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr
      1540              1545                  1550                  1555
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     GTT CGT CGG TAT CCG GCC ACT GGT GCC AAT GGC CAG CTG ATG TCC AAC   960
     Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn
                      1560                1565                1570

 5   GCT TTG CCT TCA GGA CTC GAG CTG AGA GAC AGC AGC CCA CAG GCA GCA  1008
     Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala
                      1575                1580                1585

10   CAT GTG AAC CAT CTC TCA CCC CCC TGG GGG CTG AAC CTC ACC GAG AAA  1056
     His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys
                      1590                1595                1600

     ATC AAG AAA ATC AAA GTC GTC TTC ACC CCC ACC ATC TGC AAG CAG ACC  1104
     Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr
15                    1605                1610                1615

     TGT GCC CGG GGA CGC TGT GCC AAC AGC TGT GAG AAG GGT GAC ACC ACC  1152
     Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr
                      1620                1625                1630                1635

20   ACC TTG TAC AGT CAG GGT GGC CAT GGG CAT GAC CCC AAG TCT GGC TTC  1200
     Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe
                      1640                1645                1650
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CGT ATC TAT TTC TGC CAA ATC CCC TGC CTG AAT GGT GGC CGC TGC ATC   1248
Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile
            1655                    1660                    1665

 5  GGC CGG GAC GAG TGC TGG TGT CCA GCC AAC TCC ACA GGA AAG TTC TGC   1296
    Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys
                1670                    1675                    1680

10  CAT CTG CCT GTC CCG CAG CCA GAC AGG GAA CCT GCA GGG CGA GGT TCC   1344
    His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser
                1685                    1690                    1695

    CGG CAC AGA ACC CTG CTG GAA GGT CCC CTG AAG CAA TCC ACC TTC ACG   1392
    Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr
15  1700                    1705                    1710            1715

    CTG CCT CTC TCT AAC CAG CTC GCC TCT GTG AAC CCC TCG CTG GTG AAG   1440
    Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys
                1720                    1725                    1730

20  GTG CAA ATT CAT CAC CCG CCT GAG GCC TCT GTG CAG ATT CAC CAG GTG   1488
    Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val
                1735                    1740                    1745
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GCC CGG GTC CGG GGT GAG CTG GAC CCC GTG CTG GAG GAC AAC AGT GTG      1536
Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val
      1750                            1755                      1760

GAG ACC AGA GCC TCT CAT CGC CCC CAC GGC AAC CTA GGC CAC AGC CCC      1584
Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro
      1765                      1770                      1775

TGG GCC AGC AAC AGC ATA CCC GCT CGG GCC GGA GAG GCC CCT CGG CCA      1632
Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro
      1780                      1785                      1790      1795

CCA CCA GTG CTG TCT AGG CAT TAT GGA CTT CTG GGC CAG TGT TAC CTG      1680
Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu
      1800                      1805                      1810

AGC ACG GTG AAT GGA CAG TGT GCT AAC CCC CTA GGT AGT CTG ACT TCT      1728
Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser
      1815                      1820                      1825

CAG GAG GAC TGC TGT GGC AGT GTG GGG ACC TTC TGG GGG GTG ACC TCC      1776
Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser
      1830                      1835                      1840
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     TGT GCT CCC TGC CCA CCC AGA CAA GAG GGT CCA GCC TTC CCA GTG ATT    1824
     Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile
         1845                    1850                    1855

 5   GAA AAT GGC CAG CTG GAG TGT CCC CAA GGA TAC AAG AGA CTG AAC CTC    1872
     Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu
         1860                    1865                    1870        1875

10   AGC CAC TGC CAA GAT ATC AAT GAG TGC CTG ACC CTG GGC CTC TGC AAG    1920
     Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys
             1880                    1885                    1890

     GAC TCG GAG TGC GTG AAC ACC AGG GGC AGC TAC CTG TGC ACC TGC AGG    1968
     Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg
15               1895                    1900                    1905

     CCT GGC CTC ATG CTG GAT CCG TCA AGG AGC CGC TGC GTA TCG GAC AAG    2016
     Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys
20               1910                    1915                    1920

     GCT GTC TCC ATG CAG CAG GGA CTA TGC TAC CGG TCA CTG GGG TCT GGT    2064
     Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly
                 1925                    1930                    1935
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      ACC TGC ACC CTG CCT TTG GTT CAT CGG ATC ACC AAG CAG ATA TGC TGC    2112
      Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys
      1940                    1945                    1950            1955

5     TGC AGC CGT GTG GGC AAA GCC TGG GGT AGC ACA TGT GAA CAG TGT CCC    2160
      Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro
                      1960                    1965                    1970

10    CTG CCT GGC ACA GAA GCC TTC AGG GAG ATC TGC CCT GCT GGC CAT GGC    2208
      Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly
                      1975                    1980                    1985

      TAC ACC TAC TCG AGC TCA GAC ATC CGC CTG TCT ATG AGG AAA GCC GAA    2256
      Tyr Thr Tyr Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu
                      1990                    1995                    2000

15    GAA GAG GAA CTG GCT AGC CCC TTA AGG GAG ACA GAG CAG AGC ACT        2304
      Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr
                      2005                    2010                    2015

20.   GCA CCC CCA CCT GGG CAA GCA GAG AGG CAA CCA CTC CGG GCA GCC ACC    2352
      Ala Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr
                      2020                    2025                    2030            2035
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5.   GCC ACC TGG ATT GAG GCT GAG ACC CTC CCT GAC AAA GGT GAC TCT CGG   2400
     Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg
                      2040                2045                2050

     GCT GTT CAG ATC ACA ACC AGT GCT CCC CAC CTA CCT GCC CGG GTA CCA   2448
     Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro
                      2055                2060                2065

10   GGG GAT GCC ACT GGA AGA CCA GCA CCA TCC TTG CCT GGA CAG GGC ATT   2496
     Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile
                      2070                2075                2080

     CCA GAG AGT CCA GCA GAA GAG CAA GTG ATT CCC TCC AGT GAT GTC TTG   2544
     Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu
                      2085                2090                2095

15   GTG ACA CAC AGC CCC CCA GAC TTT GAT CCA TGT TTT GCT GGA GCC TCC   2592
     Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser
                      2100                2105                2110      2115

20   AAC ATC TGT GGC CCT GGG ACC TGT GTG AGC CTC CCA AAT GGA TAC AGA   2640
     Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg
                      2120                2125                2130
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TGT GTC TGC AGC CCT GGC TAC CAG CTA CAC CCC AGC CAA GAC TAC TGT      2688
Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys
                    2135                2140                2145

ACT GAT GAC AAC GAG TGT ATG AGG AAC CCC TGT GAA GGA AGA GGG CGC      2736
Thr Asp Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg
    2150                2155                2160

TGT GTC AAC AGT GTG GGC TCC TAC TCC TGC CTC TGC TAT CCT GGC TAC      2784
Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr
                    2165                2170                2175

ACA CTA GTC ACC CTC GGA GAC ACA CAG GAG TGC CAA GAT ATC GAT GAG      2832
Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu
2180                2185                2190                2195

TGT GAG CAG CCC GGG GTG TGC AGT GGT GGG CGA TGC AGC AAC ACG GAG      2880
Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu
        2200                2205                2210

GGC TCG TAC CAC TGC GAG TGT GAT CGG GGC TAC ATC ATG GTC AGG AAA      2928
Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys
            2215                2220                2225
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     GGA CAC TGT CAA GAT ATC AAC GAA TGC CGT CAC CCT GGT ACC TGC CCT     2976
     Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro
                           2230                        2240

5    GAT GGG AGA TGC GTC AAC TCC CCT GGC TCC TAC ACT TGT CTG GCC TGT     3024
     Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys
               2245                    2250                    2255

10   GAG GAG GGC TAT GTA GGC CAG AGT GGG AGC TGT GAT GTC AAT GAG         3072
     Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu
          2260                    2265                2270           2275

15   TGT CTG ACC CCT GGG ATA TGT ACC CAT GGA AGG TGC ATC AAC ATG GAA     3120
     Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu
                    2280                         2285                2290

20   GGC TCC TTT AGA TGC TCC TGT GAG CCG GGC TAT GAG GTC ACC CCA GAC     3168
     Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp
                         2295                    2300           2305

     AAG AAG GGC TGC CGA GAT GTG GAC GAG TGT GCC AGC CGA GCC TCG TGC     3216
     Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys
               2310                    2315                    2320
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                                                                              3264
      CCC ACG GGC CTC TGC CTC AAC ACG GAG GGC TCC TTC ACC TGC TCA GCC
      Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala
          2325          2330                    2335

                                                                              3312
  5   TGT CAG AGC GGG TAC TGG GTG AAC GAA GAT GGC ACT GCC TGT GAA GAC
      Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Ala Cys Glu Asp
          2340          2345                2350                 2355

                                                                              3360
 10   TTG GAT GAA TGT GCC TTC CCT GGA GTC TGC CCC ACA GGC GTC TGC ACC
      Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr
          2360          2365                2370

                                                                              3408
 15   AAT ACT GTA GGC TCC TTC TCC TGC AAG GAC TGT GAC CAG GGC TAC CGG
      Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg
          2375          2380                2385

                                                                              3456
      CCC AAC CCC CTG GGC AAC AGA TGC GAA GAT GTG GAT GAG TGT GAA GGT
      Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly
          2390          2395                2400

                                                                              3504
 20   CCC CAA AGC AGC TGC CGG GGA GGC GAA TGC AAG AAC ACA GAA GGT TCC
      Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser
          2405          2410                2415
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5   TAC CAA TGC CTC TGT CAC CAG GGC TTC CAG CTG GTC AAT GGC ACC ATG   3552
   Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met
   2420               2425               2430               2435

   TGT GAG GAC GTG AAT GAG TGT GTT GGG GAA GAG CAT TGT GCT CCT CAC   3600
   Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His
   2440               2445               2450

10  GGC GAG TGC CTC AAC AGC CTG GGC TCC TTC TTC TGC CTC TGT GCA CCC   3648
   Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro
   2455               2460               2465

   GGC TTT GCT AGT GCT GAG GGG GGC ACC AGA TGC CAG GAT GTT GAT GAA   3696
   Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu
   2470               2475               2480

15  TGT GCA GCC ACA GAC CCG TGT CCG GGA GGA CAC TGT GTC AAC ACA GAG   3744
   Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu
   2485               2490               2495

20  GGC TCC TTC AGC TGT CTG TGT GAG ACT GCT TCC TTC CAG CCC TCC CCA   3792
   Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro
   2500               2505               2510               2515

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      GAC AGC GGA GAA TGT TTG GAT ATT GAT GAG TGT GAG GAC CGT GAA GAC    3840
      Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp
                      2520                2525                2530

  5   CCG GTG TGC GGA GCC TGG AGG TGT GAG AAC AGT CCT GGT TCC TAC CGC    3888
      Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg
                      2535                2540                2545

 10   TGC ATC CTG GAC TGC CAG CCT GGA TTC TAT GTG GCG CCA AAT GGA GAC    3936
      Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp
                      2550                2555                2560

 15   TGC ATT GAC ATA GAT GAA TGT GCC AAT GAC ACT GTG TGT GGG AAC CAT    3984
      Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His
                      2565                2570                2575

 20   GGC TTC TGT GAC AAC ACG GAC GGC TCC TTC CGC CTG TGT GAC CAG        4032
      Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln
                      2580                2585            2590    2595

      GGC TTC GAG ACC TCA CCA TCA GGC TGG GAG TGT GTT GAT GTG AAC GAG    4080
      Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu
                      2600                2605                2610
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        TGT GAG CTC ATG ATG GCA GTG TGT GGG GAT GCG CTC TGT GAG AAC GTG          4128
        Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val
                      2615                    2620                    2625

   5    GAA GGC TCC TTC CTG TGC CTT TGC GCC AGT GAC CTT GAG GAG TAC GAC          4176
        Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp
                      2630                    2635                    2640

  10    GCA GAA GAA GGA CAC TGC CGT CCT CGG GTG GCT GGA GCT CAG AGA ATC          4224
        Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile
                      2645                    2650                    2655

        CCA GAG GTC CGG ACA GAG GAC CAG GCT CCA AGC CTT ATC CGC ATG GAA          4272
        Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu
  15    2660                    2665                    2670                    2675

        TGC TAC TCT GAA CAC AAT GGT CCT CCC TGC TCT CAA ATC CTG GGC              4320
        Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly
                      2680                    2685                    2690

  20    CAG AAC TCC ACA CAG GCC GAG TGC TGC ACT CAG GGT GCC AGA TGG              4368
        Gln Asn Ser Thr Gln Ala Glu Cys Cys Thr Gln Gly Ala Arg Trp
                      2695                    2700                    2705
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GGA AAG GCC TGT GCG CCC TGC CCA TCT GAG GAC TCA GTT GAA TTC AGT          4416
Gly Lys Ala Pro Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser
                              2710                          2715                          2720

5   CAG CTC TGC CCC AGT GGT CAA GGT TAC ATC CCA GTG GAA GGA GCC TGG          4464
    Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp
                              2725                          2730                          2735

10  ACA TTT GGA CAA ACC ATG TAT ACA GAT GCC GAT GAA TGT GTA CTG TTT          4512
    Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe
                              2740                          2745                          2750   2755

    GGG CCT GCT CTC TGC CAG AAT GGC CGA TGC TCA AAC ATA GTG CCT GGC          4560
    Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly
15                            2760                          2765                          2770

    TAC ATT TGC CTG TGC AAC CCT GGC TAC CAC TAT GAT GCC TCC AGC AGG          4608
    Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg
                              2775                          2780                          2785

20  AAG TGC CAG GAT CAC AAC GAA TGC CAG GAC TTG GCC TGT GAG AAC GGT          4656
    Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly
                              2790                          2795                          2800

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      GAG TGT GTG AAC CAA GAA GGC TCC TTC CAT TGC CTC TGC AAT CCC CCC   4704
      Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro
      2805                    2810                    2815

 5    CTC ACC CTA GAC CTC AGT GGG CAG CGC TGT GTG AAC ACG ACC AGC AGC   4752
      Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser
      2820                    2825                    2830          2835

      ACG GAG GAC TTC CCT GAC CAT GAC ATC CAC ATG GAC ATC TGC TGG AAA   4800
10    Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys
      2840                    2845                    2850

      AAA GTC ACC AAT GAT GTG TGC AGC CAG CCC TTG CGT GGG CAC CAT ACC   4848
      Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr
15    2855                    2860                    2865

      ACC TAT ACA GAA TGC TGC CAA GAT GGG GAG GCC TGG AGC CAG CAA       4896
      Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln
      2870                    2875                    2880

20    TGC GCT CTG TGC CCG CCC AGG AGC TCT GAG GTC TAC GCT CAG CTG TGC   4944
      Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys
      2885                    2890                    2895
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                                                                          4992
   AAC GTG GCT CGG ATT GAG GCA GAG CGC GGA GCA GGG ATC CAC TTC CGG
   Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg
   2900              2905              2910              2915

                                                                          5040
5  CCA GGC TAT GAG TAT GGC CCT GGC CTG GAC GAT CTG CCT GAA AAC CTC
   Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu
                     2920              2925              2930

                                                                          5088
10 TAC GGC CCA GAT GGG GCT CCC TTC TAT AAC TAC CTA GGC CCC GAG GAC
   Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp
                     2935              2940              2945

                                                                          5136
   ACT GCC CCT GAG CCT CCC TTC TCC AAC CCA GCC AGC CAG CCG GGA GAC
   Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp
15                   2950              2955              2960

                                                                          5184
   AAC ACA CCT GTC CTT GAG CCT CCT CTG CAG CCC TCT GAA CTT CAG CCT
   Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro
20                   2965              2970              2975

                                                                          5232
   CAC TAT CTA GCC AGC CAC TCA GAA CCC CCT GCC TCC TTC GAA GGC CTT
   His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu
                     2980              2985              2990       2995
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      CAG GCT GAG GAA TGT GGC ATC CTG AAT GGC TGT GAG AAT GGC CGC TGC   5280
      Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys
              3000                        3005                  3010

 5    GTG CGT GTG CGG GAG GGC TAC ACT TGC GAC TGC TTT GAG GGC TTC CAG   5328
      Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln
              3015                        3020                  3025

      CTG GAT GCG CCC ACA TTG GCC TGT GTG GAT GTG AAC GAG TGT GAA GAC   5376
      Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp
10            3030                        3035                  3040

      TTG AAC GGG CCT GCA CGA CTC TGT GCA CAC GGT CAC TGT GAG AAC ACA   5424
      Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr
15            3045                        3050                  3055

      GAG GGT TCC TAT CGC TGC CAC TGT TCG CCA GGT TAC GTG GCA GAG CCA   5472
      Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro
              3060                        3065                  3070        3075
20
      GGC CCC CCA CAC TGT GCG GCC AAG GAG TAG                            5502
      Gly Pro Pro His Cys Ala Ala Lys Glu  *
              3080                        3085
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      Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His
                              70                  75              80

      Pro Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp
  5           85                      90              95

      Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln
 10                   100                 105             110

      Gly Asn Pro Gly Trp Leu Ala Glu Ala Arg Arg Pro Pro Arg
                      115                 120             125

      Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser
 15                   130                 135             140

      His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Ala Pro Ser Val
                      145                 150             155         160

      Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Arg Arg Gly Arg
 20                   165                 170             175

      Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr
                      180                 185             190
```

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 1834 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser
1               5                   10                  15

His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys
            20                  25                  30

Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val
        35                  40                  45

Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu
    50                  55                  60

Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro
195                 200                 205

Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg
5       210                 215                 220

Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu
225                 230                 235                 240

Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg
10      245                 250                 255

Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr
260                 265                 270

Arg Ile Gln Pro Leu Val Pro Pro Ser Pro Pro Ser Arg Arg
15      275                 280                 285

Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr
290                 295                 300

Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn
20      305                 310                 315                 320

Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala
325 330 335

His Val Asn His Leu Ser Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys
340 345 350

5

Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr
355 360 365

10 Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr
370 375 380

Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe
385 390 395 400

15

Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile
405 410 415

Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys
420 425 430

20

His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser
435 440 445

```
    Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr
                450                 455                 460

5   Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys
                465                 470                 475         480

    Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val
                485                 490                 495

10  Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val
                500                 505                 510

    Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro
                515                 520                 525

15  Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro
                530                 535                 540

    Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu
                545                 550                 555         560

20  Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser
                565                 570                 575
```

```
        Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser
                    580                     585                     590

        Cys Ala Pro Cys Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile
    5               595                     600                     605

        Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu
   10               610                     615                     620

        Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys
   15               625                     630                     635     640

        Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg
                    645                     650                     655

        Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys
   15               660                     665                     670

        Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly
   20               675                     680                     685

        Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys
                    690                     695                     700
```

Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro
705                     710                     715                     720

Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly
                        725                     730                     735

Tyr Thr Tyr Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu
                        740                     745                     750

10   Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr
                        755                     760                     765

Ala Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr
                        770                     775                     780

Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg
785                     790                     795                     800

Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro
                        805                     810                     815

20   Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile
                        820                     825                     830

```
      Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu
                          835                 840                     845

5     Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser
                      850                     855                 860

10    Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg
                  865                     870                 875             880

      Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys
                      885                 890                     895

15    Thr Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg
                  900                     905                     910

      Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr
                  915                     920                     925

20    Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu
                  930                     935                     940

      Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu
                  945                 950                     955             960
```

Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys
965                                        970                     975

5   Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro
        980                          985                          990

    Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys
            995                         1000                    1005

10  Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu
        1010                        1015                    1020

    Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu
    1025                        1030                    1035        1040

15  Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp
                1045                        1050                    1055

    Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys
                        1060                        1065            1070

20  Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala
                    1075                        1080                1085

```
        Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp
        1090                    1095                    1100

5       Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr
        1105                    1110                    1115                    1120

        Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg
        1125                    1130                    1135

10      Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly
        1140                    1145                    1150

        Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser
        1155                    1160                    1165

15      Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met
        1170                    1175                    1180

        Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His
20      1185                    1190                    1195                    1200

        Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro
        1205                    1210                    1215
```

Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu
1220                               1225                          1230

5   Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu
1235                               1240                     1245

Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro
1250                               1255                          1260

10  Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp
1265                               1270                     1275                1280

Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg
1285                               1290                     1295

15  Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp
1300                               1305                          1310

Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His
1315                               1320                     1325

20  Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln
1330                               1335                     1340

Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu
1345                       1350                      1355                      1360

Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val
1365                       1370                      1375

5

Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp
1380                       1385                      1390

10    Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile
1395                       1400                      1405

Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu
1410                       1415                      1420

15

Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly
1425                       1430                      1435                      1440

Gln Asn Ser Thr Gln Ala Glu Cys Cys Cys Thr Gln Gly Ala Arg Trp
1445                       1450                      1455

20

Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser
1460                       1465                      1470

Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp
1475                    1480                    1485

Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe
5                       1490                    1495                    1500

Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly
1505                    1510                    1515                    1520

10  Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg
1525                    1530                    1535

Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly
1540                    1545                    1550

15  Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro
1555                    1560                    1565

Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser
20  1570                    1575                    1580

Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys
1585                    1590                    1595                    1600

Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr
                    1605                    1610                    1615

Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln
                    1620                    1625                    1630

Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys
                    1635                    1640                    1645

10      Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg
                    1650                    1655                    1660

        Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu
                    1665                    1670                    1675                    1680

15      Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp
                    1685                    1690                    1695

        Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp
                    1700                    1705                    1710

20      Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro
                    1715                    1720                    1725

His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu
1730                             1735                         1740

Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys
5       1745                         1750                        1755                1760

Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln
1765                            1770                       1775

10  Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp
1780                        1785                              1790

Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr
1795                         1800                          1805

15  Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro
1810                        1815                            1820

Gly Pro Pro His Cys Ala Ala Lys Glu *
20  1825                           1830

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## CLAIMS

1.   A method for transferring a nucleic acid segment
into bone progenitor cells, comprising contacting bone
progenitor cells with a composition comprising an
isolated nucleic acid segment so as to transfer said
nucleic acid segment into said cells.


2.   The method of claim 1, wherein said cells are
located within a bone progenitor tissue site of an animal
and said tissue site is contacted with said composition
so as to promote nucleic acid transfer into bone
progenitor cells *in situ*.


3.   The method of claim 2, wherein the contacting
process comprises bringing said isolated nucleic acid
segment into contact with a bone-compatible matrix to
form a matrix-nucleic acid segment composition and
bringing said matrix-nucleic acid segment composition
into contact with said tissue site.


4.   The use of a composition comprising an isolated
nucleic acid segment and a bone-compatible matrix in the
preparation of a formulation or medicament for
transferring a nucleic acid segment into bone progenitor
cells.


5.   A use according to claim 4, wherein said formulation
or medicament is intended for use in transferring a
nucleic acid segment into bone progenitor cells within a
bone progenitor tissue site of an animal.

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6.   A use according to claim 5, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment formulation or medicament intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

7.   A use according to claim 6, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrix-nucleic acid segment formulation or medicament.

8.   A use according to claim 6, wherein said formulation or medicament further comprises a detectable agent for use in an imaging modality.

9.   A use according to claim 8, wherein said formulation or medicament further comprises a radiographic agent.

10.  A use according to claim 8, wherein said formulation or medicament further comprises a paramagnetic ion.

11.  A use according to claim 8, wherein said formulation or medicament further comprises a radioactive ion.

12.  A use according to claim 4, wherein said nucleic acid segment is a DNA molecule.

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13.  A use according to claim 4, wherein said nucleic acid segment is an RNA molecule.

5  14.  A use according to claim 4, wherein said nucleic acid segment is an antisense nucleic acid molecule.

15.  A use according to claim 4, wherein said nucleic
10  acid segment is a linear nucleic acid molecule, a plasmid, a recombinant insert within the genome of a recombinant virus, or a nucleic acid segment associated with a liposome.

15

16.  A use according to claim 15, wherein said nucleic acid segment is a nucleic acid segment associated with a liposome.

20

17.  A use according to claim 4, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

25

18.  A use according to claim 6, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic
30  ester polymer or lactic acid polymer matrix.

19.  A use according to claim 18, wherein said bone-compatible matrix is a titanium matrix.

35

20. A use according to claim 19, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

21. A use according to claim 18, wherein said bone-compatible matrix is a collagen preparation.

22. A use according to claim 21, wherein said bone-compatible matrix is a type II collagen preparation.

23. A use according to claim 22, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

24. A use according to claim 22, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

25. A use according to claim 22, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

26. A method of stimulating bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated osteotropic gene so as to promote expression of said gene in said cells.

27. The method of claim 26, wherein said cells are located within a bone progenitor tissue site of an animal

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and said tissue site is contacted with said composition
so as to promote bone tissue growth.


5    28.   The method of claim 27, wherein the contacting
process comprises bringing said osteotropic gene into
contact with a bone-compatible matrix to form a matrix-
gene composition and bringing said matrix-gene
composition into contact with said tissue site.

10


29.   The use of a composition comprising an isolated
osteotropic gene in the preparation of a formulation or
medicament for use in promoting expression of the gene in
15   bone progenitor cells and for stimulating said bone
progenitor cells.


30.   A use according to claim 29, wherein said
20   formulation or medicament is intended for use in
promoting expression of the gene in bone progenitor cells
within a bone progenitor tissue site of an animal and for
stimulating said bone progenitor cells to promote bone
tissue growth.

25


31.   A use according to claim 30, wherein said
formulation or medicament is prepared by bringing an
osteotropic gene into contact with a bone-compatible
30   matrix to form a matrix-gene formulation or medicament
intended for use in promoting expression of the gene in
bone progenitor cells within a bone progenitor tissue
site of an animal and for stimulating said bone
progenitor cells to promote bone tissue growth.

35

32. A use according to claim 31, wherein said
formulation or medicament is prepared by bringing an
osteotropic gene into contact with a bone-compatible
matrix and a pluronic agent to form a syringeable matrix-
gene formulation or medicament.

33. A use according to claim 31, wherein said
formulation or medicament further comprises a detectable
agent for use in an imaging modality.

34. A use according to claim 33, wherein said
formulation or medicament further comprises a
radiographic agent.

35. A use according to claim 34, wherein said
formulation or medicament further comprises calcium
phosphate.

36. A use according to claim 33, wherein said
formulation or medicament further comprises a
paramagnetic ion.

37. A use according to claim 36, wherein said
formulation or medicament further comprises chromium
(III), manganese (II), iron (III), iron (II), cobalt
(II), nickel (II), copper (II), neodymium (III), samarium
(III), ytterbium (III), gadolinium (III), vanadium (II),
terbium (III), dysprosium (III), holmium (III) or
erbium (III).

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38. A use according to claim 33, wherein said
formulation or medicament further comprises a radioactive
ion.

5

39. A use according to claim 38, wherein said
formulation or medicament further comprises iodine[131],
iodine[123], technicium[99m], indium[111], rhenium[188], rhenium[186],
gallium[67], copper[67], yttrium[90], iodine[125] or astatine[211].

10

40. A use according to claim 29, wherein said
osteotropic gene is in the form of plasmid DNA, a DNA
insert within the genome of a recombinant adenovirus, a

15   DNA insert within the genome of a recombinant adeno-
associated virus (AAV), a DNA insert within the genome of
a recombinant retrovirus, or a DNA segment associated
with a liposome.

20

41. A use according to claim 40, wherein said
osteotropic gene is in the form of an osteotropic gene
associated with a liposome.

25

42. A use according to claim 29, wherein said
osteotropic gene is a parathyroid hormone (PTH) gene, a
bone morphogenetic protein (BMP) gene, a growth factor
gene, a growth factor receptor gene, a cytokine gene or a

30   chemotactic factor gene.

43. A use according to claim 42, wherein said
osteotropic gene is a transforming growth factor (TGF)

35   gene, a fibroblast growth factor (FGF) gene, a
granulocyte/macrophage colony stimulating factor (GMCSF)

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gene, an epidermal growth factor (EGF) gene, a platelet derived growth factor (PDGF) gene, an insulin-like growth factor (IGF) gene, or a leukemia inhibitory factor (LIF) gene.

44.    A use according to claim 43, wherein said osteotropic gene is a TGF-$\alpha$, TGF-$\beta$1 or TGF-$\beta$2 gene.

45.    A use according to claim 42, wherein said osteotropic gene is a PTH gene.

46.    A use according to claim 42, wherein said osteotropic gene is a BMP gene.

47.    A use according to claim 46, wherein said osteotropic gene is a BMP-2 or BMP-4 gene.

48.    A use according to claim 31, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

49.    A use according to claim 48, wherein said bone-compatible matrix is a titanium matrix.

50.    A use according to claim 49, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

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51.  A use according to claim 48, wherein said bone-
compatible matrix is a collagen preparation.


5    52.  A use according to claim 51, wherein said bone-
compatible matrix is a type II collagen preparation.


53.  A use according to claim 52, wherein said bone-
10   compatible matrix is a type II collagen preparation
obtained from hyaline cartilage.


54.  A use according to claim 52, wherein said bone-
15   compatible matrix is a recombinant type II collagen
preparation.


55.  A use according to claim 52, wherein said bone-
20   compatible matrix is a mineralized type II collagen
preparation.


56.  A use according to claim 31, wherein said matrix-
25   gene composition is applied to a bone fracture site in
said animal.


57.  A use according to claim 31, wherein said matrix-
30   gene composition is implanted within a bone cavity site
in said animal.


58.  A use according to claim 31, wherein said bone
cavity site is the result of dental or periodontal
35   surgery or the removal of an osteosarcoma.

59.   A composition comprising an isolated nucleic acid
segment in association with a bone-compatible matrix.


60.   The composition of claim 59, wherein said nucleic
acid segment is a DNA molecule.


61.   The composition of claim 59, wherein said nucleic
acid segment is an RNA molecule.


62.   The composition of claim 59, wherein said nucleic
acid segment is an antisense nucleic acid molecule.


63.   The composition of claim 59, wherein said nucleic
acid segment is a linear nucleic acid molecule, a
plasmid, a recombinant insert within the genome of a
recombinant virus, or a nucleic acid segment associated
with a liposome.


64.   The composition of claim 63, wherein said nucleic
acid segment is associated with a liposome.


65.   The composition of claim 59, wherein said nucleic
acid segment encodes a polypeptide or protein that
stimulates bone progenitor cells when expressed in said
cells.


66.   The composition of claim 59, wherein said bone-
compatible matrix is a collagenous, titanium,
hydroxylapatite, hydroxylapatite-coated titanium,
bioglass, aluminate, bioceramic, acrylic ester polymer or

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lactic acid polymer matrix.


67.   The composition of claim 66, wherein said bone-
compatible matrix is a collagen preparation.


68.   The composition of claim 67, wherein said bone-
compatible matrix is a type II collagen preparation.


69.   The composition of claim 68, wherein said bone-
compatible matrix is a type II collagen preparation
obtained from hyaline cartilage.


70.   The composition of claim 68, wherein said bone-
compatible matrix is a recombinant type II collagen
preparation.


71.   The composition of claim 68, wherein said bone-
compatible matrix is a mineralized type II collagen
preparation.


72.   The composition of claim 59, further defined as a
syringeable composition.


73.   The composition of claim 59, wherein said
composition further comprises a detectable agent for use
in an imaging modality.

74. The composition of claim 73, wherein said composition further comprises a radiographic agent.

5    75. The composition of claim 73, wherein said composition further comprises a paramagnetic ion.

76. The composition of claim 73, wherein said
10   composition further comprises a radioactive ion.

77. A composition comprising an isolated osteotropic gene in association with a bone-compatible matrix, said composition being capable of stimulating bone growth when
15   administered to a bone progenitor tissue site of an animal.

78. The composition of claim 77, wherein said
20   osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated
25   with a liposome.

79. The composition of claim 78, wherein said osteotropic gene is in the form of an osteotropic gene
30   associated with a liposome.

80. The composition of claim 77, wherein said osteotropic gene is a PTH, BMP, TGF-$\alpha$, TGF-$\beta$1, TGF-$\beta$2,
35   FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

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81.    The composition claim 80, wherein said osteotropic
gene is a TGF-α, TGF-β1, TGF-β2, PTH, BMP-2 or BMP-4
gene.

5

82.    The composition of claim 77, wherein said bone-
compatible matrix is a collagenous, metal,
hydroxyapatite, bioglass, aluminate, bioceramic, acrylic
ester polymer or lactic acid polymer matrix.

10

83.    The composition of claim 82, wherein said bone-
compatible matrix is a titanium matrix.

15

84.    The composition of claim 83, wherein said bone-
compatible matrix is a titanium matrix coated with
hydroxylapatite.

20

85.    The composition of claim 82, wherein said bone-
compatible matrix is a collagen preparation.

25     86.    The composition of claim 85, wherein said bone-
compatible matrix is a type II collagen preparation.

87.    The composition of claim 86, wherein said bone-
30     compatible matrix is a type II collagen preparation
obtained from hyaline cartilage.

88.    The composition of claim 86, wherein said bone-
compatible matrix is a recombinant type II collagen
35     preparation.

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89.   The composition of claim 86, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

5

90.   The composition of claim 77, further defined as comprising an isolated osteotropic gene in association with a bone-compatible matrix and a pluronic agent, the composition forming a syringeable composition.

10

91.   The composition of claim 77, wherein said composition further comprises a detectable agent for use in an imaging modality.

15

92.   The composition of claim 91, wherein said composition further comprises a radiographic agent.

20

93.   The composition of claim 92, wherein said composition further comprises calcium phosphate.

25   94.   The composition of claim 91, wherein said composition further comprises a paramagnetic ion.

95.   The composition of claim 94, wherein said
30   composition further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

35

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96.  The composition of claim 91, wherein said composition further comprises a radioactive ion.

5    97.   The composition of claim 96, wherein said composition further comprises iodine[131], iodine[123], technicium[99m], indium[111], rhenium[188], rhenium[186], gallium[67], copper[67], yttrium[90], iodine[125] or astatine[211].

10

98.   A kit comprising, in suitable container means, a pharmaceutically acceptable bone-compatible matrix and a pharmaceutically acceptable osteotropic gene preparation.

15

99.   The kit of claim 98, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer
20   matrix.

100. The kit of claim 99, wherein said bone-compatible matrix is a titanium matrix.
25

101. The kit of claim 99, wherein said bone-compatible matrix is a hydroxylapatite-coated titanium matrix.

30   102. The kit of claim 99, wherein said bone-compatible matrix is a collagenous matrix.

103. The kit of claim 102, wherein said bone-compatible
35   matrix is a type II collagen matrix.

104. The kit of claim 103, wherein said bone-compatible matrix is a type II collagen matrix obtained from hyaline cartilage.

5

105. The kit of claim 103, wherein said bone-compatible matrix is a recombinant type II collagen matrix.

10      106. The kit of claim 103, wherein said bone-compatible matrix is a mineralized type II collagen matrix.

107. The kit of claim 98, wherein said osteotropic gene
15      preparation comprises a linear osteotropic gene, a plasmid including an osteotropic gene, a recombinant virus having a genome that includes an osteotropic gene or an osteotropic gene associated with a liposome.

20

108. The kit of claim 98, wherein said osteotropic gene preparation comprises a lyophilized gene preparation.

25      109. The kit of claim 98, wherein said osteotropic gene preparation comprises a PTH, TGF, BMP, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

30      110. The kit of claim 109, wherein said osteotropic gene preparation comprises a PTH, TGF-ß1, TGF-ß2, TGF-ß3, BMP-2 or a BMP-4 gene.

35      111. The kit of claim 98, further comprising a pluronic agent.

- 264 -

112. The kit of claim 98, further comprising a detectable agent for use in an imaging modality.

5

113. The kit of claim 112, wherein said composition further comprises a radiographic agent.

10    114. The kit of claim 113, wherein said composition further comprises calcium phosphate.

115. The kit of claim 112, wherein said composition
15    further comprises a paramagnetic ion.

116. The kit of claim 115, wherein said composition further comprises chromium (III), manganese (II), iron
20    (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

25

117. The kit of claim 112, wherein said composition further comprises a radioactive ion.

30    118. The kit of claim 117, wherein said composition further comprises iodine[131], iodine[123], technicium[99m], indium[111], rhenium[188], rhenium[186], gallium[67], copper[67], yttrium[90], iodine[125] or astatine[211].

35

119. The kit of claim 98, wherein said bone-compatible

- 265 -

matrix and said osteotropic gene preparation are present within a single container means.

5    120. The kit of claim 119, wherein said container means is a syringe or pipette.

121. The kit of claim 98, wherein said bone-compatible
10   matrix and said osteotropic gene preparation are present within distinct container means.

122. The kit of claim 98, further comprising a third
15   container means comprising a pharmaceutically acceptable diluent.

123. The kit of claim 98, further comprising a syringe, pipette or forceps.
20

124. An osteotropic device, comprising an isolated osteotropic gene capable of expression in bone progenitor cells, the gene associated with an amount of a bone-
25   compatible matrix effective to absorb said gene, wherein said device is capable of stimulating bone formation when implanted within a bone progenitor tissue site of an animal.

30

125. The device of claim 124, wherein said device is a titanium or a hydroxylapatite-coated titanium device.

35   126. The device of claim 124, wherein said device is shaped to join a bone fracture site in said animal.

- 266 -

127. The device of claim 124, wherein said device is shaped to fill a bone cavity site in said animal.

5

128. The device of claim 124, wherein said device is an artificial joint.

10    129. A DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3.

15

130. The DNA segment of claim 129, comprising an isolated gene that includes a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2.

20

131. A composition comprising a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3.

25

132. A method for stimulating a bone progenitor cell, comprising contacting a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen.

30

133. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for stimulating a bone

35    progenitor cell.

134. A use according to claim 133, wherein said composition comprises type II collagen purified from hyaline cartilage.

5

135. A use according to claim 133, wherein said composition comprises recombinant type II collagen.

10  136. A use according to claim 133, wherein said composition comprises type II collagen further supplemented with minerals.

15  137. A use according to claim 136, wherein said composition comprises type II collagen further supplemented with calcium.

20  138. A use according to claim 133, wherein said composition comprises between about 1 mg and about 500 mg of type II collagen.

25  139. A use according to claim 138, wherein said composition comprises between about 1 mg and about 100 mg of type II collagen.

30  140. A use according to claim 139, wherein said composition comprises about 10 mg of type II collagen.

141. A use according to claim 133, wherein said
35  composition comprises type II collagen in combination with a nucleic acid segment that encodes a polypeptide or

- 268 -

protein that stimulates bone progenitor cells when
expressed in said cells.

5    142. A use according to claim 141, wherein said nucleic
acid segment comprises an isolated PTH gene, BMP gene,
growth factor gene, growth factor receptor gene, cytokine
gene or chemotactic factor gene.

10

143. A use according to claim 142, wherein said nucleic
acid segment comprises an isolated BMP gene.

15    144. A use according to claim 143, wherein said nucleic
acid segment comprises an isolated BMP-2 or BMP-4 gene.

145. A use according to claim 141, wherein said
20   composition further comprises a detectable agent for use
in an imaging modality.

146. A use according to claim 133, wherein said
25   formulation or medicament is intended for use in
stimulating a bone progenitor cell located within a bone
progenitor tissue site of an animal and for promoting
bone tissue growth.

30

147. A use according to claim 146, wherein said
formulation or medicament is intended for use in
implantation within a bone cavity site in an animal and
for promoting bone tissue growth in said bone cavity
35   site.

- 269 -

148. A use according to claim 146, wherein said
formulation or medicament is intended for use in
implantation within a bone fracture site in an animal and
for promoting bone tissue growth in said bone fracture
5    site.


149. A method for promoting bone growth, comprising
contacting a bone progenitor tissue site of an animal
10   with a composition comprising type II collagen in an
amount effective to activate bone progenitor cells of
said tissue site.


15   150. The use of a composition comprising a biologically
effective amount of type II collagen in the preparation
of a formulation or medicament for promoting bone growth
in a bone progenitor tissue site of an animal.

20
151. A use according to claim 150, wherein said
composition comprises recombinant type II collagen.


25   152. A use according to claim 150, wherein said
composition comprises type II collagen further
supplemented with minerals.


30   153. A use according to claim 150, wherein said
composition comprises type II collagen and an osteotropic
gene in a combined amount effective to activate bone
progenitor cells of said tissue site.


35

154. A use according to claim 153, wherein said composition comprises type II collagen in combination with a PTH, TGF-ß or BMP gene.

5

155. A use according to claim 153, wherein said composition further comprises a detectable agent for use in an imaging modality.
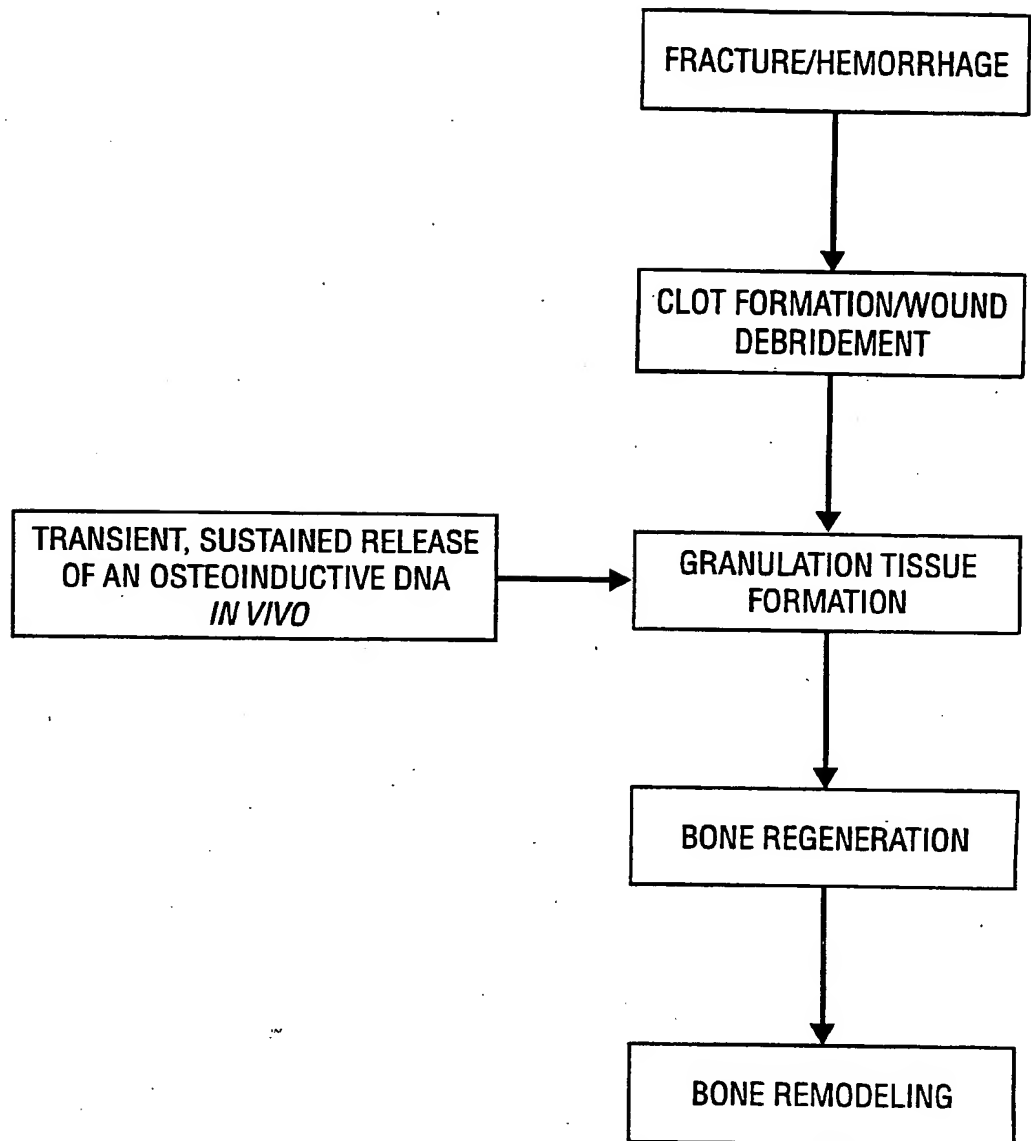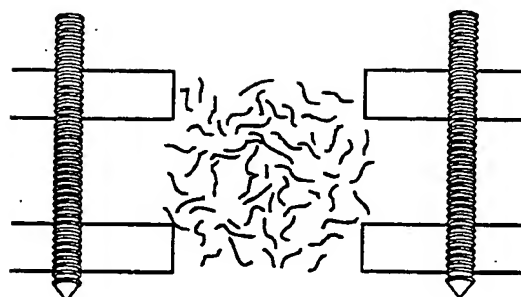
FIG. 1

FIG. 2A
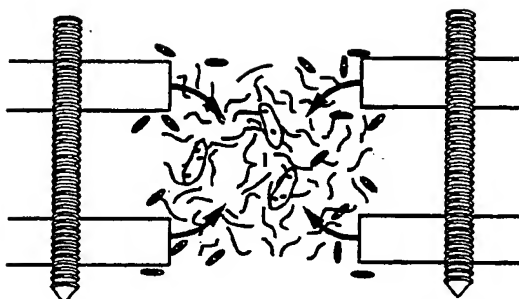
FIG. 2B

FIG. 2C

FIG. 2D
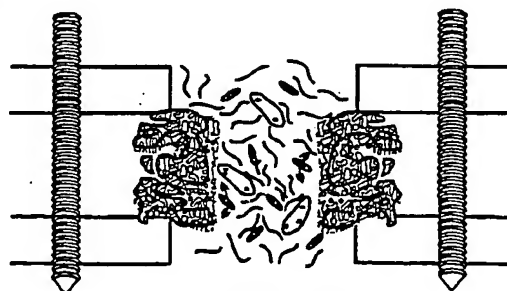
FIG. 2E
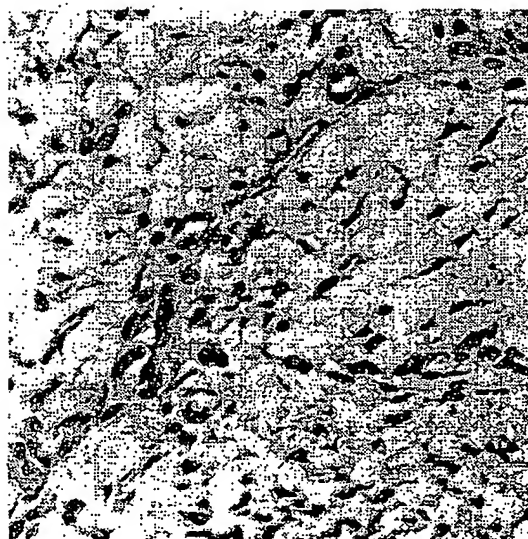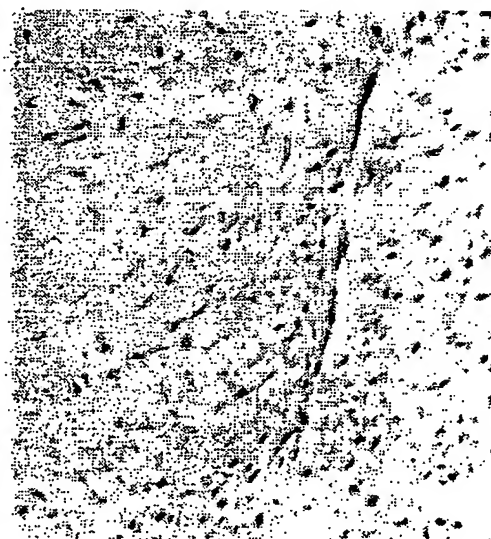
FIG. 3A          FIG. 3B          FIG. 3C



FIG.3D                    FIG. 3E
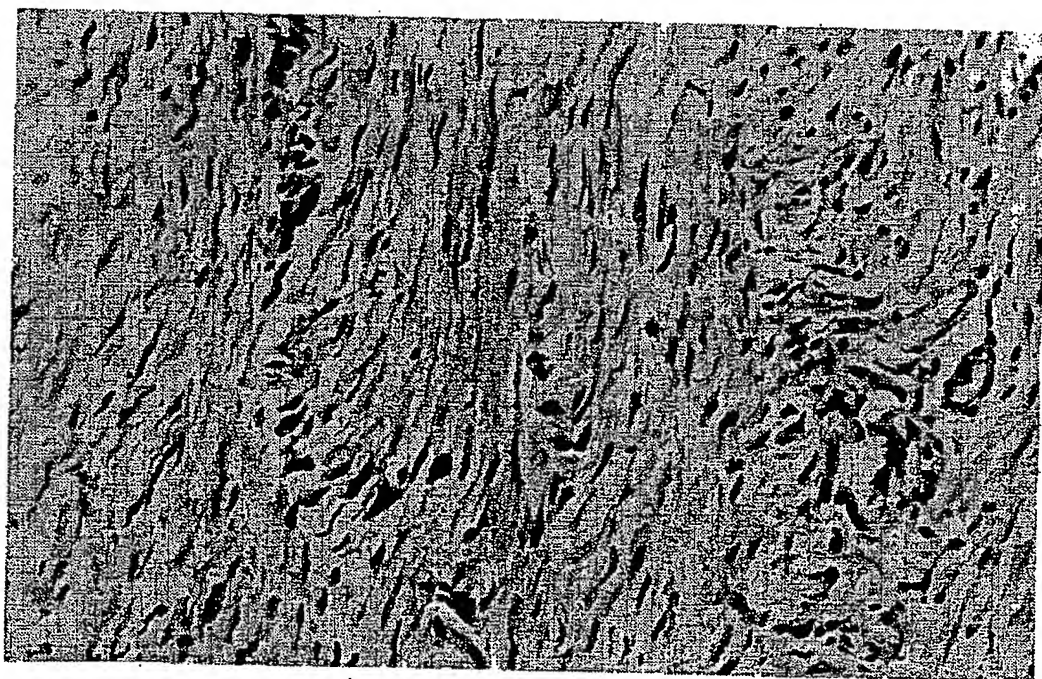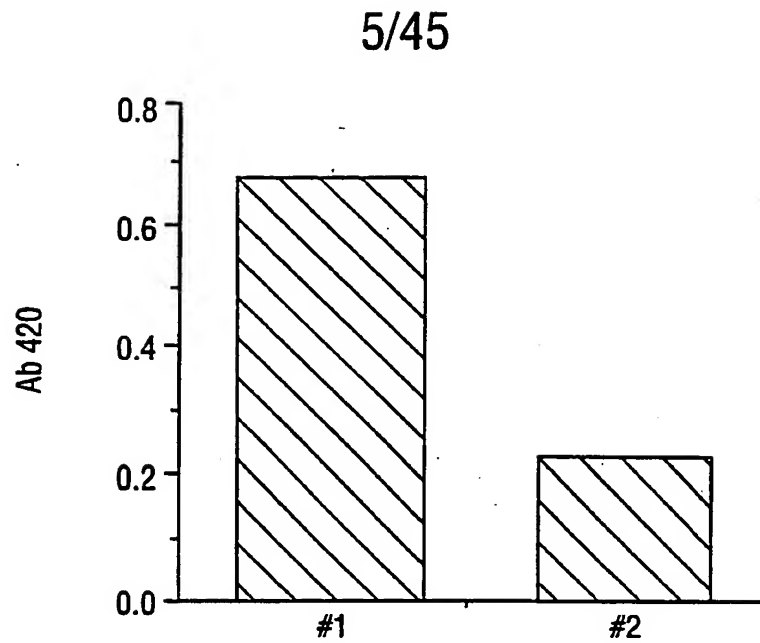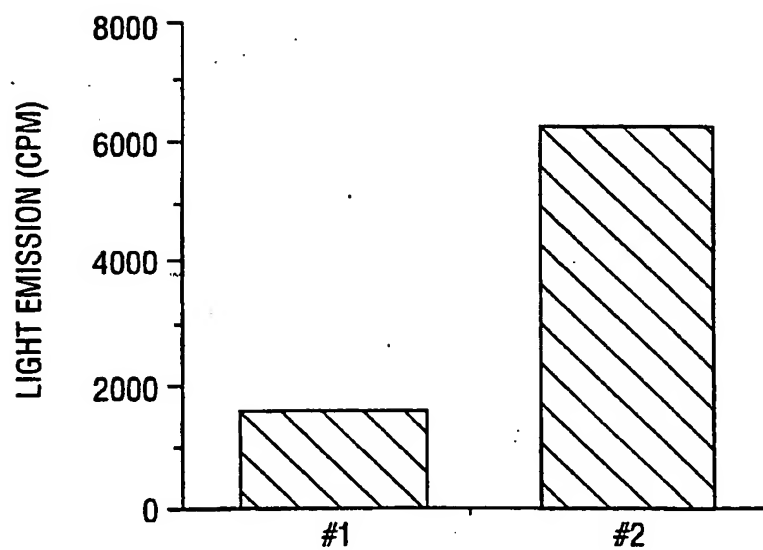
FIG. 4

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FIG. 5A



FIG. 5B

FIG. 6A



FIG. 6B



FIG. 6C
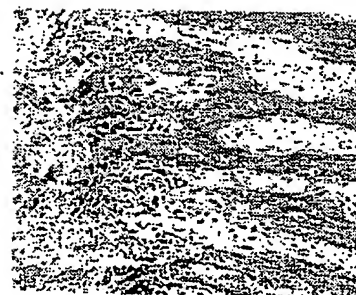


FIG. 6D
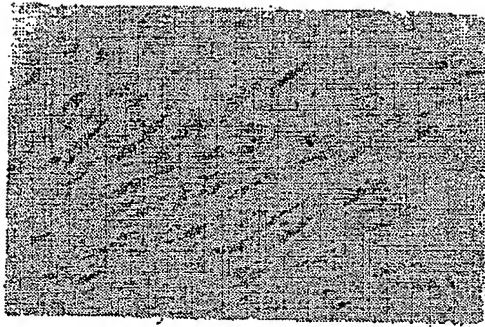
FIG. 7A



FIG. 7B
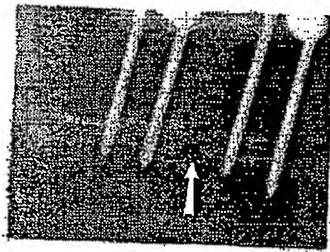
FIG. 8A
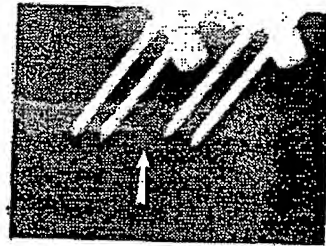


FIG. 8B



FIG. 8C
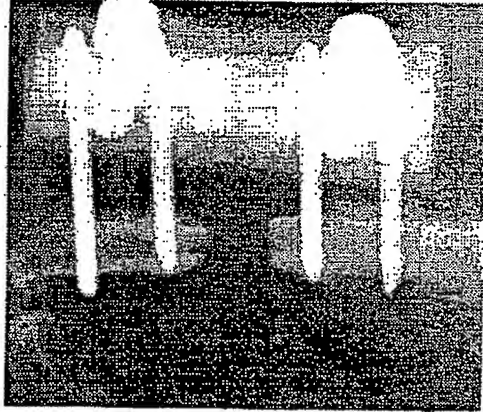
FIG. 9A
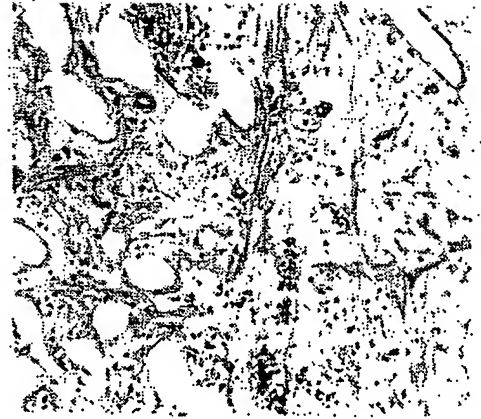


FIG. 9B
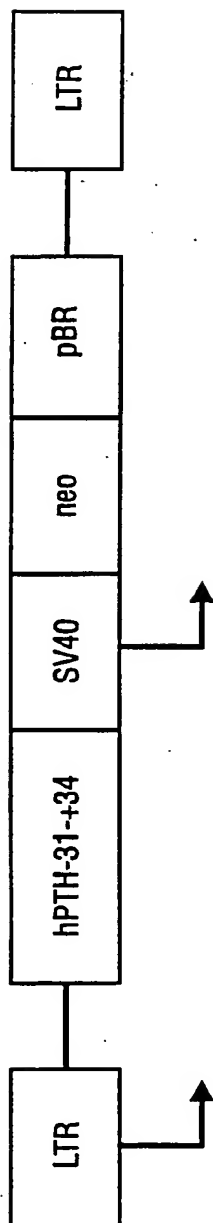
PLJ-hPTH 1-34

FIG. 10

1 · 2 · 3 · 4

4.3—

FIG. 11

4.4 —                    **hPTH1-34**

7.5 —                    **β-gal**

                            **Neo**

4.4 —

2.4 —                    **β-actin**

# FIG. 12

CONTROL
FEMUR

OSTEOTOMY
FEMUR

FIG. 13
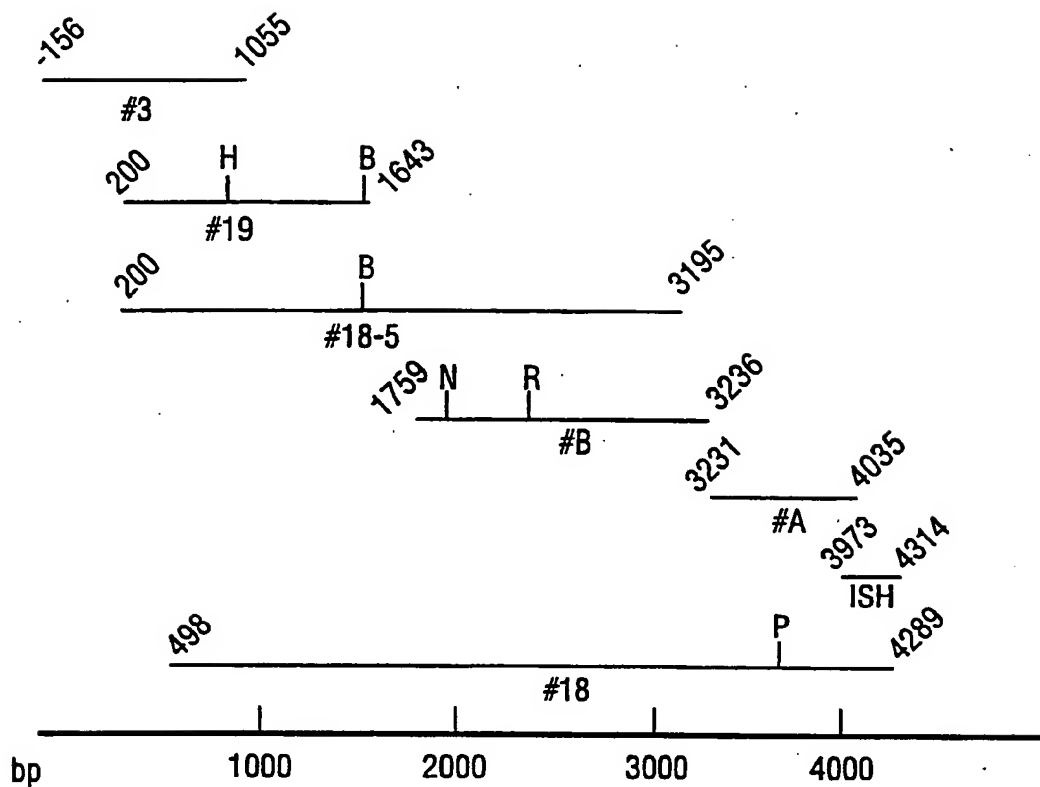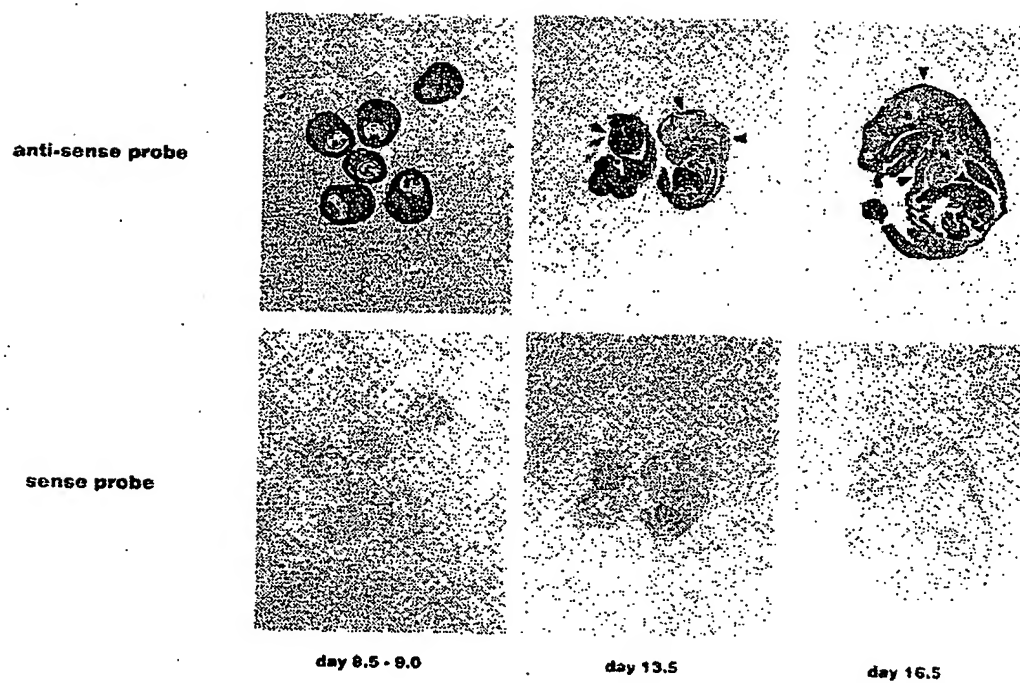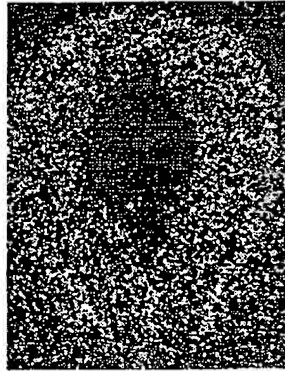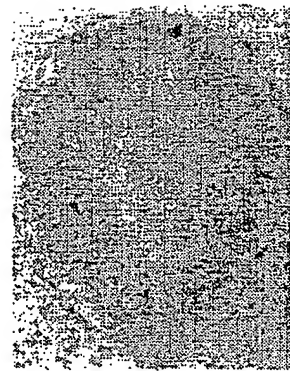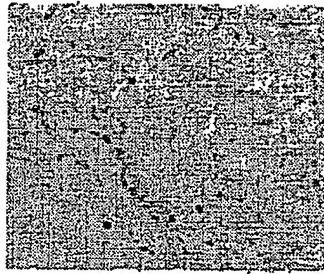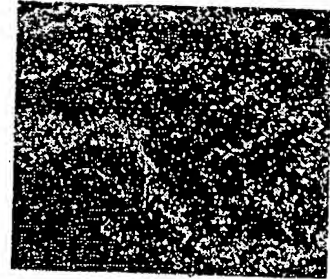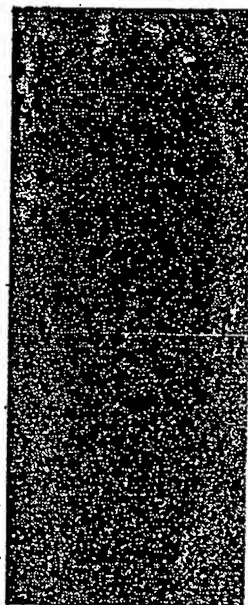
FIG. 14

FIG. 15A



FIG. 15B



FIG. 15C

anti-sense probe

sense probe

day 8.5 - 9.0     day 13.5     day 16.5

## FIG. 16

FIG. 17A



FIG. 17B

FIG. 17C



FIG. 17D
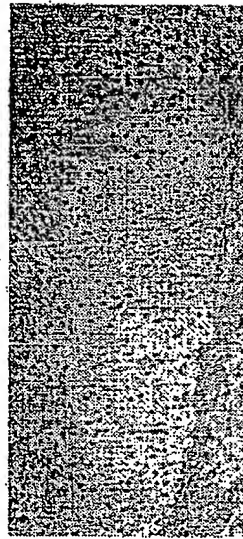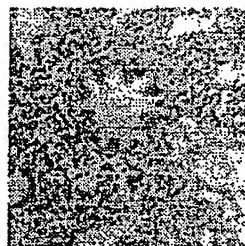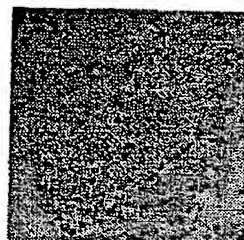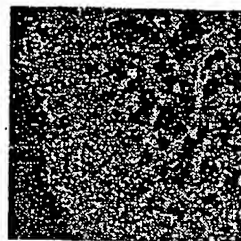
FIG. 18A

FIG. 18B
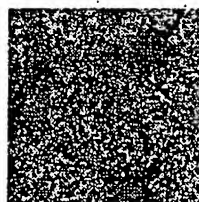
FIG. 18C
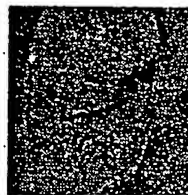


FIG. 18D

FIG. 18E



FIG. 18F

FIG. 18G



FIG. 18H
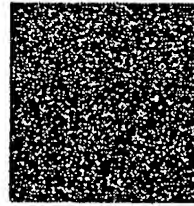
FIG. 18I



FIG. 18J
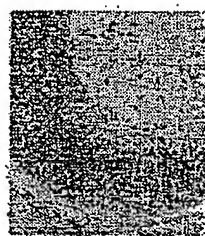
FIG. 18K



FIG. 18L
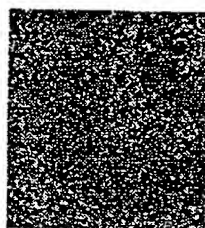
FIG. 18M

FIG. 18N

FiG. 180

FIG. 18P

4.4 kb—
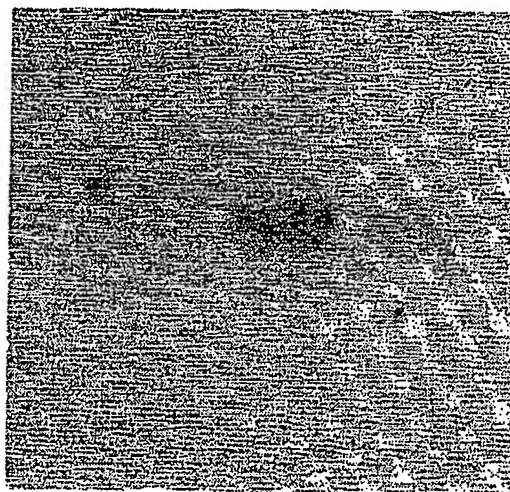
DAY            5      14      28

FIG. 19
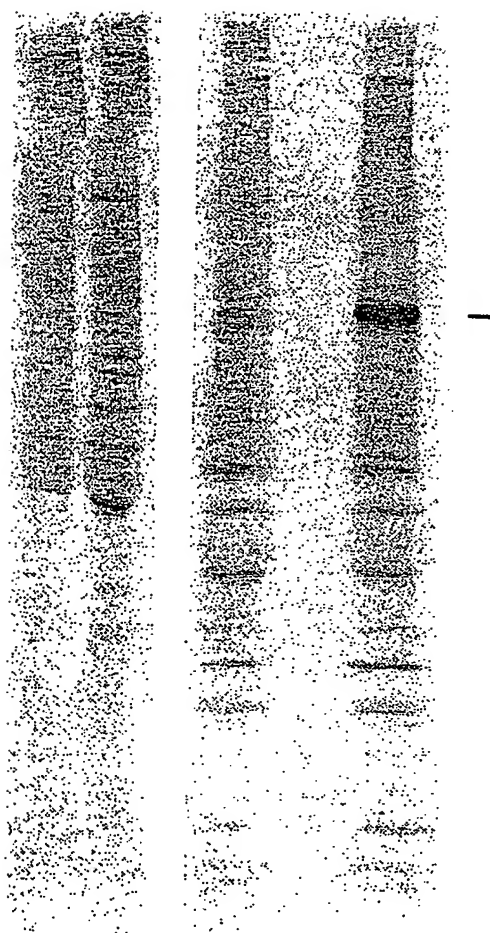
FIG. 20
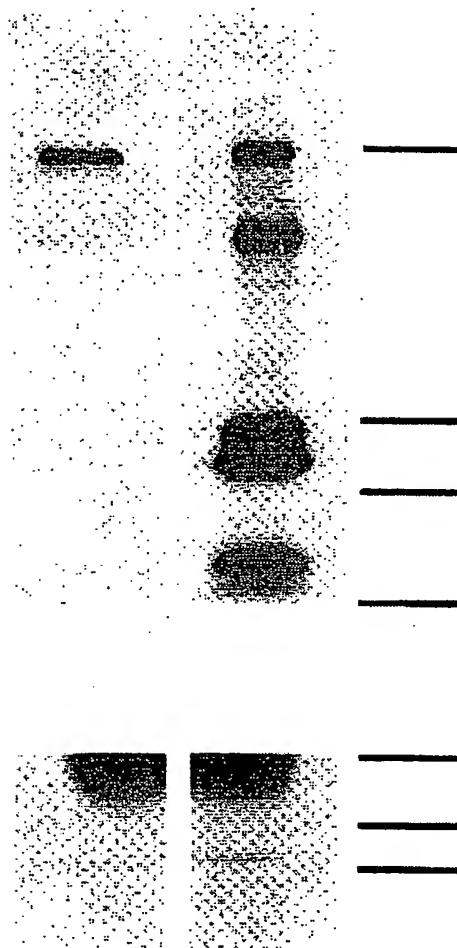
FIG. 21
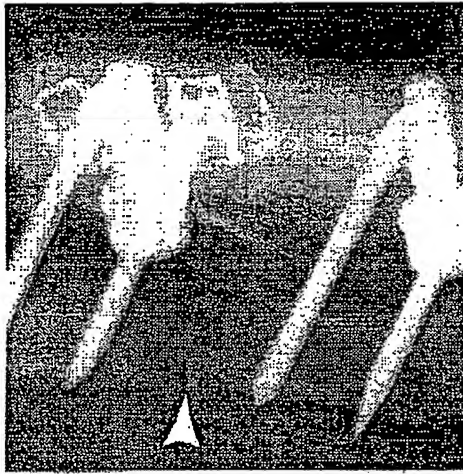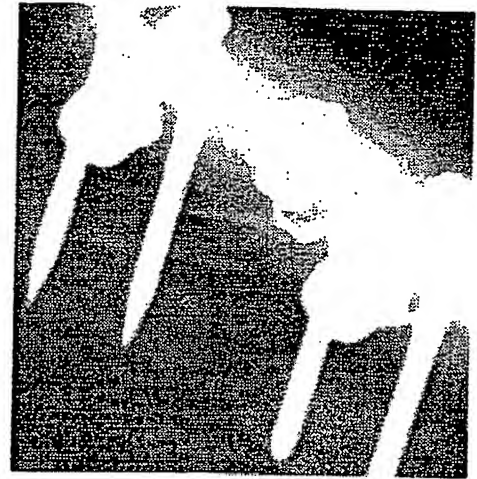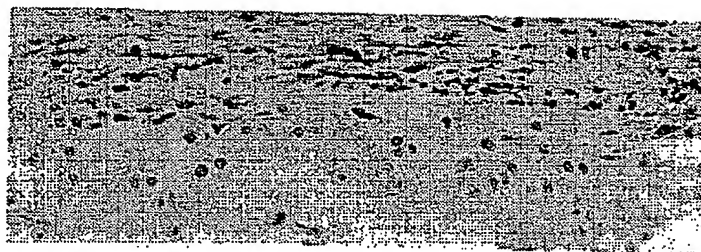
FIG. 22A



FIG. 22B



FIG. 22C

FIG. 23A



FIG. 23B



FIG. 23C

MIPGNRMLMV VLLCQVLLGG ATDASLMPET GKKKVAEIQG HAGGRRSGQS HELLRDFEAT LLQMFGLRRR
PQPSKSAVIP DYMSDLYRLQ SGEEEEEQS QGTGLEYPER PASSANTVSS FHHEEHLENI PGTSESSAFR
FFFNLSSIPE NEVISSAELR LFREQVDQGP DWEQGFHRMN IYEVMKPPAE MVPGHLITRL LDTSLVRHNV
TRWETFDVSP AVLRWTREKQ PNYGLAIEVT HLHQTRTHQG QHVSISRSLP QGSGNWAQLR PLLVTFGHDG
RGHTLTRRSA KRSPKHHPQR SSKKNKNCRR HSLYVDFSDV GWNDWIVAPP GYQAFYCHGD CPFPLADHLN
STNHAIVQTL VNSVNSSIPK ACCVPTELSA ISMLYLDEYD KVVLKNYQEM VVEGCGCRYP YDVPDYA

FIG. 24

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```
ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG CTG GGC 54
 M   R   Q   A   A   L   G   L   L   A   L   L   L   L   A   L   L   G   18

CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG GCG GGG GCG GGG 108
 P   G   G   R   G   V   G   R   P   G   S   G   A   Q   A   G   A   G   36

CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT GTG ATC TGC AAG CGG ACC 162
 R   W   A   Q   R   F   K   V   V   F   A   P   V   I   C   K   R   T   54

TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT CAG CAG GGC TCC AAC ATG ACG CTC 216
 C   L   K   G   Q   C   R   D   S   C   Q   Q   G   S   N   M   T   L   72

ATC GGA GAG AAC GGC CAC AGC ACC GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG 270
 I   G   E   N   G   H   S   T   D   T   L   T   G   S   A   F   R   V   90

GTG GTG TGC CCT CTA CCC TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG 324
 V   V   C   P   L   P   C   M   N   G   G   Q   C   S   S   R   N   Q   108

TGC CTG TGT CCC CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA 378
 C   L   C   P   P   D   F   T   G   R   F   C   Q   V   P   A   A   G   126

ACC GGA GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC 432
 T   G   A   G   T   G   S   S   G   P   G   W   P   D   R   A   M   S   144

ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC AAA CAC 486
 T   G   P   L   P   P   L   A   P   E   G   E   S   V   A   S   K   H   162

GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG GGG GAG GGT CCT 540
 A   I   Y   A   V   Q   V   I   A   D   P   P   G   P   G   E   G   P   180

CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG CCA GGA CAA ATC TCG GCA 594
 P   A   Q   H   A   A   F   L   V   P   L   G   P   G   Q   I   S   A   198

GAA GTG CAG GCT CCG CCC CCC GTG GTG AAC GTG CGT GTC CAT CAC CCT CCT GAA 648
 E   V   Q   A   P   P   P   V   V   N   V   R   V   H   H   P   P   E   216

GCT TCC GTT CAG GTG CAC CGC ATC GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT 702
 A   S   V   Q   V   H   R   I   E   G   P   N   A   E   G   P   A   S   234

TCC CAG CAC TTG CTG CCG CAT CCC AAG CCC CAG CAC CCG AGG CCA CCC ACT CAA 756
 S   Q   H   L   L   P   H   P   K   P   Q   H   P   R   P   P   T   Q   252

AAG CCA CTG GGC CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC 810
 K   P   L   G   R   C   F   Q   D   T   L   P   K   Q   P   C   G   S   270

AAC CCT TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT 864
 N   P   L   P   G   L   T   K   Q   E   D   C   C   G   S   I   G   T   288

GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA GGG GTG 918
 A   W   G   Q   S   K   C   H   K   C   P   Q   L   Q   Y   T   G   V   306

CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC CCC CAG GGC TAC 972
 Q   K   P   V   P   V   R   G   E   V   G   A   D   C   P   Q   G   Y   324

AAG AGG CTC AAC AGC ACC CAC TGC CAG GAT ATC AAC GAA TGT GCG ATG CCC GGG 1026
 K   R   L   N   S   T   H   C   Q   D   I   N   E   C   A   M   P   G   342
```

# FIG. 25-1

**SUBSTITUTE SHEET (RULE 26)**

## 36/45

```
AAT GTG TGC CAT GGT GAC TGC CTC AAC AAC CCT GGC TCT TAT CGC TGT GTC TGC 1080
 N   V   C   H   G   D   C   L   N   N   P   G   S   Y   R   C   V   C  360

CCG CCC GGT CAT AGC TTG GGT CCC CTC GCA GCA CAG TGC ATT GCC GAC AAA CCA 1134
 P   P   G   H   S   L   G   P   L   A   A   Q   C   I   A   D   K   P  378

GAG GAG AAG AGC CTG TGT TTC CGC CTT GTG AGC ACC GAA CAC CAG TGC CAG CAC 1188
 E   E   K   S   L   C   F   R   L   V   S   T   E   H   Q   C   Q   H  396

CCT CTG ACC ACA CGC CTA ACC CGC CAG CTC TGC TGC TGT AGT GTG GGT AAA GCC 1242
 P   L   T   T   R   L   T   R   Q   L   C   C   C   S   V   G   K   A  414

TGG GGT GCC CGG TGC CAG CGC TGC CCG GCA GAT GGT ACA GCA GCC TTC AAG GAG 1296
 W   G   A   R   C   Q   R   C   P   A   D   G   T   A   A   F   K   E  432

ATC TGC CCC GGC TGG GAA AGG GTA CCA TAT CCT CAC CTC CCA CCA GAC GCT CAC 1350
 I   C   P   G   W   E   R   V   P   Y   P   H   L   P   P   D   A   H  450

CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC GGG CCA CCC AAA 1404
 H   P   G   G   K   R   L   L   P   L   P   A   P   D   G   P   P   K  468

CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCA CCC CTC GAG GAC ACA GAG 1458
 P   Q   Q   L   P   E   S   P   S   R   A   P   P   L   E   D   T   E  486

GAA GAG AGA GGA GTG ACC ATG GAT CCA CCA GTG AGT GAG GAG CGA TCG GTG CAG 1512
 E   E   R   G   V   T   M   D   P   P   V   S   E   E   R   S   V   Q  504

CAG AGC CAC CCC ACT ACC ACC ACC TCA CCC CCC CGG CCT TAC CCA GAG CTC ATC 1566
 Q   S   H   P   T   T   T   T   S   P   P   R   P   Y   P   E   L   I  522

TCT CGC CCC TCC CCA CCT ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC 1620
 S   R   P   S   P   P   T   F   H   R   F   L   P   D   L   P   P   S  540

CGA AGT GCA GTG GAG ATC GCC CCC ACT CAG GTC ACA GAG ACC GAT GAG TGC CGA 1674
 R   S   A   V   E   I   A   P   T   Q   V   T   E   T   D   E   C   R  558

TTG AAC CAG AAT ATC TGT GGC CAT GGA CAG TGT GTG CCT GGC CCC TCG GAT TAC 1728
 L   N   Q   N   I   C   G   H   G   Q   C   V   P   G   P   S   D   Y  576

TCC TGC CAC TGC AAC GCT GGC TAC CGG TCA CAC CCG CAG CAC CGC TAC TGT GTT 1782
 S   C   H   C   N   A   G   Y   R   S   H   P   Q   H   R   Y   C   V  594

GAT GTG AAC GAG TGC GAG GCA GAG CCC TGC GGC CCC GGG AAA GGC ATC TGT ATG 1836
 D   V   N   E   C   E   A   E   P   C   G   P   G   K   G   I   C   M  612

AAC ACT GGT GGC TCC TAC AAT TGT CAC TGC AAC CGA GGC TAC CGC CTC CAC GTG 1890
 N   T   G   G   S   Y   N   C   H   C   N   R   G   Y   R   L   H   V  630

GGT GCA GGG GGC CGC TCG TGC GTG GAC CTG AAC GAG TGC GCC AAG CCT CAC CTG 1944
 G   A   G   G   R   S   C   V   D   L   N   E   C   A   K   P   H   L  648

TGT GGG GAC GGT GGC TTC TGC ATC AAC TTC CCT GGT CAC TAC AAA TGC AAC TGC 1998
 C   G   D   G   G   F   C   I   N   F   P   G   H   Y   K   C   N   C  666

TAT CCT GGC TAC CGG CTC AAG GCC TCC CGA CCG CCC ATT TGC GAA GAC ATC GAC 2052
 Y   P   G   Y   R   L   K   A   S   R   P   P   I   C   E   D   I   D  684

GAG TGT CGC GAC CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA CCT GGC 2106
 E   C   R   D   P   S   T   C   P   D   G   K   C   E   N   K   P   G  702
```

# FIG. 25-2

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```
AGC TTC AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC 2160
 S   F   K   C   I   A   C   Q   P   G   Y   R   S   Q   G   G   G   A  720

TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA TGG TGT 2214
 C   R   D   V   N   E   C   S   E   G   T   P   C   S   P   G   W   C  738

GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG ATA CGA ACC CGC 2268
 E   K   L   P   G   S   Y   R   C   T   C   A   Q   G   I   R   T   R  756

ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT GAG GCT GGG AAA GTG TGC 2322
 T   G   R   L   S   C   I   D   V   D   D   C   E   A   G   K   V   C  774

CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC TCT TTC CAG TGT CAG TGC CTC TCC 2376
 Q   D   G   I   C   T   N   T   P   G   S   F   Q   C   Q   C   L   S  792

GGC TAT CAT CTG TCA AGG GAT CGG AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC 2430
 G   Y   H   L   S   R   D   R   S   R   C   E   D   I   D   E   C   D  810

TTC CCT GCG GCC TGC ATC GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA 2484
 F   P   A   A   C   I   G   G   D   C   I   N   T   N̲   G̲   S̲   Y   R  828

TGT CTC TGT CCC CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT 2538
 C   L   C   P   L   G   H   R   L   V   G   G   R   K   C   K   K   D  846

ATA GAT GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC 2592
 I   D   E   C   S   Q   D   P   G   L   C   L   P   H   A   C   E   N  864

CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC CAG GAC 2646
 L   Q   G   S   Y   V   C   V   C   D   E   G   F   T   L   T   Q   D  882

CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG GAG TGC TAC CTT 2700
 Q   H   G   C   E   E   V   E   Q   P   H   H   K   K   E   C   Y   L  900

AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG GCT ACC AAT GTC ACT CAG 2754
 N   F   D   D   T   V   F   C   D   S   V   L   A   T   N̲   V̲   T̲   Q  918

CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC TGG GGA GAC CAC TGC GAA ATC TAT 2808
 Q   E   C   C   C   S   L   G   A   G   W   G   D   H   C   E   I   Y  936

CCC TGT CCA GTC TAC AGC TCA GCC GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA 2862
 P   C   P   V   Y   S   S   A   E   F   H   S   L   V   P   D   G   K  954

AGG CTA CAC TCA GGA CAA CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC 2916
 R   L   H   S   G   Q   Q   H   C   E   L   C   I   P   A   H   R   D  972

ATC GAC GAA TGC ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG 2970
 I   D   E   C   I   L   F   G   A   E   I   C   K   E   G   K   C   V  990

AAC TCG CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024
 N   S   Q   P   G   Y   E   C   Y   C   K   Q   G   F   Y   Y   D   G  1008

AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC TGC AGG 3078
 N   L   L   E   C   V   D   V   D   E   C   L   D   E   S   N   C   R  1026

AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC ACT CCG CCG GCA 3132
 N   G   V   C   E   N   T   W   R   L   P   C   A   C   T   P   P   A  1044

GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG AGC CCG GAG GAG ATG GAG CAC GCC 3186
 E   Y   S   P   A   Q   A   Q   C   L   S   P   E   E   M   E   H   A  1062
```

## FIG. 25-3

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```
CCA GAG AGA CGT GAA GTG TGC TGG GGC CAG CGA GGA GAG GAC GGC ATG TGT ATG 3240
 P   E   R   R   E   V   C   W   G   Q   R   G   E   D   G   M   C   M  1080

GGG CCC CTG GCG GGA CCT GCC CTC ACT TTT GAT GAC TGC TGC TGC CGC CAG CCG 3294
 G   P   L   A   G   P   A   L   T   F   D   D   C   C   C   R   Q   P  1098

CGG CTG GGG TAC CAG TGC AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC 3348
 R   L   G   Y   Q   C   R   P   C   P   P   R   G   T   G   S   Q   C  1116

CCG ACT TCA CAG AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG 3402
 P   T   S   Q   S   E   S   N   S   F   W   D   T   S   P   L   L   L  1134

GGG AAG TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT 3456
 G   K   S   P   R   D   E   D   S   S   E   E   D   S   D   E   C   R  1152

TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG TGT CCT 3510
 C   V   S   G   P   C   V   P   R   P   G   G   A   V   C   E   C   P  1170

GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC ATT GAT GAG TGC 3564
 G   G   F   Q   L   D   A   S   R   A   R   C   V   D   I   D   E   C  1188

CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC GAG CGG TGC GTG AAC ACC 3618
 R   E   L   N   Q   R   G   L   L   C   K   S   E   R   C   V   N̲   T̲  1206

AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT GGC TTC ACG CGC AGC CGC CCT CAC 3672
 S̲   G   S   F   R   C   V   C   K   A   G   F   T   R   S   R   P   H  1224

GGG CCT GCG TGC CTC AGC GCC GCC GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA 3726
 G   P   A   C   L   S   A   A   A   D   D   A   A   I   A   H   T   S  1242

GTG ATC GAT CAT CGA GGG TAT TTT CAC TGA
 V   I   D   H   R   G   Y   F   H   *
```

# FIG. 25-4

```
Met Arg Gln Ala Ala Gly Leu Leu Leu Ala Leu Leu Leu Gly Pro Gly Gly Arg      22
Gly Val Gly Pro Gly Ser Gly Ala Gln Ala Gly Trp Ala Gln Arg Phe Lys Val      44
Val Phe Ala Pro Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Ser Cys Gln Gln  66
Gly Ser Asn Met Thr Leu Ile Gly Asn Gly Ser Thr Leu Asp Thr Gly Ser Ala Phe  88
Arg Val Val Cys Pro Leu Pro Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu  110
Cys Pro Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Ala Gly Thr Gly  132
Ser Ser Gly Pro Gly Trp Thr Pro Asp Arg Ala Met Ser Thr Gly Pro Leu Pro Ala Pro Glu  154
Gly Glu Val Ala Ser Lys Val His Ala Ile Gln Val Ile Leu Pro Pro Leu Ala Asp Pro Pro  176
Gly Glu Gly Pro Pro Ala Gln His Ala Ala Ile Pro Gly Gln Ile Ser Ala  198
Glu Val Gln Ala Pro Pro Val Val Asn Val Arg Val His His Pro Pro Glu Ala Ser Val Gln  220
Val His Arg Ile Glu Gly Leu Gly Pro Asn Ala Glu Ser Gln His Leu Leu Pro His Pro Pro  242
Lys Pro His Pro Thr Gln Pro Arg Pro Pro Leu Lys Gly Phe Gln Asp Thr Leu Pro  264
Lys Gln Pro Cys Gly Ser Asn Pro Leu Gly Leu Lys Cys His Asp Cys Cys Gly Ser Ile  286
Gly Thr Ala Trp Gly Gln Ser Lys His Cys Lys Lys Thr Gly Val Thr Gly Val Gln Lys  308
Pro Val Arg Gly Val Gly Val Gly Ala Asp Cys Pro Gln Gly Tyr Lys Leu Asn Ser Thr  330
His Cys Gln Asp Ile Asn Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn  352
Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Phe Leu Pro Leu Ala Ala Gln Cys Ile  374
Ala Asp Lys Pro Glu Glu Lys Thr Leu Lys Ser Leu Val Ser Thr Glu His Gln Gln His His  396
Pro Leu Thr Thr Arg Leu Gln Gly Thr Ala Asp Gly Lys Ala Trp Gly Ala Arg  418
Cys Gln Arg Cys Pro Ala Gly Thr Ala Phe Lys Ile Cys Pro Gly Trp Glu Arg Val  440
Pro Tyr Pro His Leu Pro Pro Asp Ala His Pro Gly Lys Leu Arg Leu Leu Pro Ala  462
Pro Asp Gly Pro Pro Lys Pro Gln Gln Gly Leu Pro Gln Leu Pro Glu Ser Arg Ala Pro Leu Glu Asp  484
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FIG. 26-1

```
Thr Glu Glu Arg Gly Val Thr Met Asp Pro Pro Val Ser Glu Glu Arg Ser Val Gln Gln Ser      506
His Pro Thr Thr Phe Leu Pro Asp Pro Arg Pro Ser Arg Ile Ala Val Glu Ile Ala Pro Thr Gln  528
Thr Phe His Arg Leu Pro Asp Pro Ser Arg Ser Ala Asn Gln Ile Ala Pro Val Pro Gly          550
Val Thr Glu Thr Asp Cys Arg Leu Asn Gln Ala Gly His Gly Ile Cys Gly Val Pro Gly          572
Pro Ser Asp Tyr Ser Cys His Ala Gly Tyr Arg Leu His Pro Gln His Arg Tyr Cys Val          594
Asp Val Asn Glu Cys Glu Ala Glu Gly Pro Cys Gly Pro Gly Ile Cys Met Asn Thr Gly Gly      616
Ser Tyr Asn Cys His Cys Asn Arg Gly Tyr Leu His Gly Ile Ala Gly Arg Ser Cys Val          638
Asp Leu Asn Glu Cys Ala Lys Gly Asn Arg Gly Leu Cys Gln Phe Gly Gly Phe Cys Ile Asn Phe Pro Gly  660
His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu Lys Ala Ser Arg Pro Ile Cys Glu Asp      682
Ile Asp Glu Cys Arg Asp Pro Ser Thr Cys Pro Gly Lys Ala Gly Gly Ala Cys Val Asn Lys Ser Phe  704
Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Pro Glu Cys Arg Asp Ala Val Asn Glu          726
Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly Trp Leu Gly Ser Pro Gly Ser Tyr Arg Cys Thr      748
Cys Ala Gln Gly Ile Arg Thr Gly Arg Gly Ile Cys Lys Ala Val Asp Cys Gly Glu Ala          770
Gly Lys Val Cys Gln Asp Gly Ile Cys Ser Asn Thr Pro Gly Ser Phe Gln Cys Gln Cys Leu Ser  792
Gly Tyr His Leu Ser Arg Asp Arg Ser Cys Glu Asp Ile Asp Glu Cys Phe Pro Ala Ala          814
Cys Ile Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro Leu Gly His Arg  836
Leu Val Gly Lys Arg Cys Lys Lys Cys Ser Gly Glu Asp Gly Ser Gln Cys Leu Gly Leu Cys Leu  858
Pro His Ala Cys Glu Asn Gly Gly Val Cys Val Asp Gly Val Asp Glu Ser Tyr Cys Arg Leu Thr  880
Gln Asp Gln Gln His Gly Cys Glu Val Glu Leu His Gln Gln Pro Lys Lys Gly Leu Cys Tyr Asn Phe  902
Asp Asp Thr Val Phe Cys Asp Ser Val Leu Ala Thr Asn Val Thr Ser Cys Tyr Cys Cys Ser      924
Leu Gly Ala Gly Trp Gly Asp Pro His Cys Glu Ile Tyr Pro Val Tyr Ser Ala Glu Phe          946
His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln Gln His Cys Glu Leu Cys Ile Pro  968
```

FIG. 26-2

```
Ala His Arg Asp Ile Asp Glu Cys Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val            990
Asn Ser Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly Asn Leu Leu Glu           1012
Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn Cys Val Glu Asn Gly Val Cys Glu Asn Trp           1034
Arg Leu Pro Cys Ala Cys Ala Cys Thr Pro Gln Arg Trp Ser Pro Pro Val Leu Cys Ile Ser Pro           1056
Glu Arg Trp Ser Thr Pro Gln Arg Gly Ala Ser Glu Ala Cys Leu Ser Gly Ala Ser Lys Ala Cys           1078
Val Trp Gly Pro Ala Leu Pro Ala Leu Thr Phe Gly Pro Thr Gly Val Leu Asp Cys Glu Arg Leu           1100
Gly Thr Gln Cys Arg Pro Cys Arg Pro Cys Arg Gly Thr Leu Lys Leu Ser Ser Gln Thr Ser Glu           1122
Ser Asn Ser Phe Asp Thr Asp Trp Ser Gln Cys Ser Gln Thr Phe Ser Gly Glu Glu Asp Ser Ser           1144
Glu Glu Asp Ser Asp Ser Cys Arg Cys Val Pro Cys Val Pro Arg Ala Arg Pro Gly Gly Ala Val           1166
Cys Glu Pro Gly Gly Phe Gly Leu Gly Arg Leu Cys Lys Val Asp Ile Ala Arg Cys Asp Glu Cys           1188
Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ala Gly Ala Cys Val Asn Thr Ser Gly Ser Phe           1210
Arg Cys Val Lys Ala Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Leu Ala Ala           1232
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly Tyr Phe His                        1251
```

FIG. 26-3

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```
ATGGAGAGCA CCTCCCCGCG AGGTCTCCGG TGCCCACAGC CTCTGGCGCC  ATGAGAGCGC CGACCACCGC   80
TCGCTGCTCC GGATGCATCC TTGGAGGGGC TTCCTGCCAC TTGTCCTGGC  TGTCTTGATG GGGACAAGTC  160
ATGCCCAACG GGATTCCATA GGGAGATACG AACCAGCTAG CAGGGATGCG  AATCGGTTGT GGGCAGCCAC  240
CCCGCAGCGG CTGCAGCCAA GGTGTACAGT CTGTTCCGAG AGCCTGACGC  GCCGGTCCCC CCTCTGAGTG  320
GAACCAGCCG GCCCAGGGGA ACCCGGGATG GCTCGCAGAG GCCGAGGCCA  TCGAACCCAG CAGTCGCGTC  400
GAGTCCAGCC ACCTGTCCAG ACTCGGAGAA GGCCGAGCCA GGGCCAGCAG  CCCGGGCTGC ACCTTCTGTC  480
GCGCGCCTGG AAACCCCTCA GCGACCCGCG GCTGCACGGC GAGGGCGGCT  CAGATAGCAG GGGGACAGTG  560
CTGCCCAGGA TGGACAACAT CAAACAGCAC ATCAAACCTG ............  AATGTCTGCG AACCGAGGCT  640
CCTGCAGCAG GCCCCAGTC  GTTCTGGCTT CCGTGGGGCG ............  TCCCTGTCAG TGAGGAGGAA  720
TTTGACCCTC AGAATGCCAG GCCTGTGCCC AGACGCTCAG ............  AGTCATCCC  GTGAGGCCAG  800
AGGAAGTCTA GTGACCAGAA TACAGCCGCT GGTACCACCA ............  CACAGAAGCA CAGCCCTGGC  880
CCCTGCAGCA GCACTCAGGG CCGTCCAGGA CAGTTCGTCG ............  GCGCCCTCAGC GATGTCCAAC  960
GCTTTGCCTT CAGGACTCGA GCTGAGAGAC AGCAGCCCAC ............  ATGGCCAGCT CCTGGGGGCT 1040
GAACCTCACC GAGAAAATCA AGAAAATCAA AGTCGTCTTC ............  CTCTCACCCC CGGGGACGCT 1120
GTGCCAACAG CTGTGAGAAG GGTGACACCA CCACCTTGTA ............  GACCTGTCCC GTCTGGCTTC 1200
CGTATCTATT TCTGCCAAAT CCCCTGCCTG AATGGTGGCC ............  GGCCATGGGC CAGCCAACTC 1280
CACAGGAAAG TTCTGCCATC TGCCTGTCCC GCAGCCAGAC ............  TGCTGGTGTC AGAACCCTGC 1360
TGGAAGGTCC CCTGAAGCAA TCCACTTCA  AGGGAACCTG ............  TTCCCGGCAC GCTGGTGAAG 1440
GTGCAAATTC ATCACCCGCC TGAGGCCTCT CTCGCCTCTG ............  TGAACCCTCG ACCCCGTGCT 1520
GGAGGACAAC AGTGTGGAGA CCAGAGCCTC ACCAGGTGGC ............  GGTGAGCTGG AGCAACAGCA 1600
TACCCGCTCG GGCCGGAGAG GCCCCTCGGC TAGGCCAACC ............  CCCCTGGGCC GTGTTACCTG 1680
AGCCACGGTGA ATGGACAGTG TGCTAACCCC GCTGTCTAGG ...........  TTCTGGCCA  TGGGGACCTT 1760
CTGGGGGGTG ACCTCCTGTG CTCCCTGCCC ACCAGACAA  ............  TGTGGCAGTG GGCCAGCTGG 1840
AGTGTCCCCA AGGATACAAG AGACTGAACC TCAGCCACTG CCAAGATATC  AATGAGTGCC TGACCCTGGG  CCTCTGCAAG 1920
```

FIG. 27-1

```
GACTCGGAGT GCGTGAACAC CAGGGGCAGC TACCTGTGCA CCTGCAGGCC TGGCCCTCATG CTGGATCCGT CAAGGAGCCG 2000
CTGCGTATCG GACAAGGCTG TCTCCATGCA GCAGGGACTA TGCTACCGGT CACTGGGGTC TGGTACCTGC ACCCTGCCTT 2080
TGGTTCATCG GATCACCAAG CAGATATGCT GCTGCAGCCG TGTGGGCAAA GCCTGGGGTA GCACATGTGA ACAGTGTCCC 2160
CTGCCTGGCA CAGAAGCCTT CAGGGGAGATC TGCCCTGCTG GCCATGGCTA CACCTACTCG AGCTCAGACA TCCGCCTGTC 2240
TATGAGGAAA GCCGAAGAAG AGGAACTGGC TAGCCCCTTA AGGGAGCAGA CAGAGCAGAG CACTGCCACCC CCACCTGGGC 2320
AAGCAGAGAG GCAACCACTC CGGGCAGCCA CCGCCACCTG GATTGAGGCT GAGACCCTCC CTGACAAAGG TGACTCTCGG 2400
GCTGTTCAGA TCACAACCAG TGCTCCCCAC CTACCTGCCC GGGTACCAGG GGATGCCACT GGAAGACCAG CACCATCCTT 2480
GCCTGGACAG GGCATTCCAG AGAGTCCAGC AGAAGAGCAA GTGATTCCCT CCAGTGATGT CTTGGTGACA CACAGCCCCC 2560
CAGACTTTGA TCCATGTTTT GCTGGAGCCT CCAACATCTG TGGCCCCTGGG ACCTGTGTGA GCCTCCCAAA TGGATACAGA 2640
TGTGTCTGCA GCCCTGGCTA CCAGCTACAC CCCAGCCAAG ACTACTGTAC TGATGACAAC GAGTGTATGA GGAACCCCTG 2720
TGAAGGAAGA GGGCGCTGTG TCAACAGTGT GGGCTCCTAC TCCTGCCTCT GCTATCCTGG GTCACACTA GTCACCCTCG 2800
GAGACACACA GGAGTGCCAA GATATCGATG AGTGTGAGCA GCCCGGGGTG TGCAGTGGTG GGCGATGCAG CAACACGGAG 2880
GGCTCGTACC ACTGCGAGTG TGATCGGGGC TACATCATGG TCAGGAAAGG ACACTGTCAA GATATCAACG AATGCCGTCA 2960
CCCTGGTACC TGCCCTGATG GGAGATGCGT CAACTCCCCT GGCTCCTACA CTTGTCTGGC CGAGATGTAG GGCTATGTAG 3040
GCCAGAGTGG GAGCTGTGTA GATGTCAATG AGTGTCTGAC CCCTGGGATA TGTACCCATG GAAGGTGCAT CAACATGGAA 3120
GGCTCCTTTA GATGCTCCTG TGAGCCGGGC TATGAGGTCA CCTCAACACG GAGGGCTGC CGAGATGTGG ACGAGTGTGC 3200
CAGCCGAGCC TCGTGCCCCA CGGGCCTCTG CCTCAACACG AGGGCTCCTC TCACCTGCTC AGCCTGTCAG AGCGGGTACT 3280
GGGTGAACGA AGATGGCACT GCCTGTGAAG ACTTGGATGA ATGTGCCTTC CCTCCGAGTCT GCCCCACAGG CGTCTGCACC 3360
AATACTGTAG GCTCCTTCTC CTGCAAGGAC TGTGACCAGG GCTACCGGCC CAACCCCCTG GGCAACAGAT GCGAAGATGT 3440
GGATGAGTGT GAAGGTCCCC AAAGCAGCTG CCGGGGAGGC AATGCAAGA ACACAGAAGG TTCCTACCAA TGCCTCTGTC 3520
ACCAGGGCTT CCAGCTGGTC AATGGCACCA TGTGTGAGGA CGTGAATGAG TGTGTTGGGG AAGAGCATTG TGCTCCTCAC 3600
GGCGAGTGCC TCAACAGCCT GGGCTCCTTC TTCTGCCTCT GTGCCACCCG CTTTGCTAGT GCTGAGGGGG GCACCAGATG 3680
CCAGGATGTT GATGAATGTG CAGCCACAGA CCCGTGTCCG GGAGGACACT GTGTCAACAC AGAGGGCTCC TTCAGCTGTC 3760
TGTGTGAGAC TGCTTCCTTC CAGCCCTCCC CAGACAGCGG AGAATGTTTG GATATTGATG AGTGTGAGGA CCGTGAAGAC 3840
```

FIG. 27-2

```
CCGGTGTGCG GAGCCTGGAG GTGTGAGAAC AGTCCTGGTT CCTACCGCTG CATCCTGGAC TGCCAGCCTG GATTCTATGT 3920
GGCGCCAAAT GGAGACTGCA TTGACATAGA TGAATGTGCC AATGACACTG TGTGTGGGAA CCATGGCTTC TGTGACAACA 4000
CGGACGGCTC CTTCCGCTGC CTGTGTGACC AGGGCTTCGA GACCTCACCA TCAGGCTGGG AGTGTGTTGA TGTGAACGAG 4080
TGTGAGCTCA TGATGGCAGT GTGTGGGGAT GCGCTCTGTG AGAACGTGGA AGGCTCCTTC CTGTGCCTTT GCGCCAGTGA 4160
CCTTGAGGAG TACGACGCAG AAGAAGGACA CTGCCGTCCT CGGGTGGCTG GAGCTCAGAG AATCCCAGAG GTCCGGACAG 4240
AGGACCAGGC TCCAAGCCTT ATCCGCATGG AATGCTACTC TGAACACAAT GGTGGTCCTC CCTGCTCTCA AATCCTGGGC 4320
CAGAACTCCA CACAGGCCGA GTGCTGCTGC ACTCAGGGTG CCAGATGGGG AAAGGCCTGT GCGCCCTGCC CATCCTGAGGA 4400
CTCAGTTGAA TTCAGTCAGC TCTGCCCCAG TGGTCAAGGT TACATCCCAG TGGAAGGAGC CTGGACATTT GGACAAACCA 4480
TGTATACAGA TGCCGATGAA TGTGTACTGT TTGGGCCTGC TCTCTGCCAG AATGGCCGAT GCTCAAACAT AGTGCCTGGC 4560
TACATTTGCC TGTGCAACCC TGGCTACCAC TATGATGCCT CCAGCAGGAA GTGCCAGGAT CACAACGAAT GCCAGGACTT 4640
GGCCCTGTGAG AACGGTGAGT GTGTGAACCA AGAAGGCTCC TTCCATTGCC TCTGCAATCC CCCCCTCACC CTAGACCTCA 4720
GTGGGCAGCG CTGTGTGAAC ACGACCAGCA GCACGGAGGA CTTCCCTGAC CATGACATCC ACATGACAT CTGCTGGAAA 4800
AAAGTCACCA ATGATGTGTG CAGCCAGCCC TTGCGTGGGC ACCATACCAC CTATACAGAA TGCTGCTGCC AAGATGGGA 4880
GGCCTGGAGC CAGCAATGCG CTCTGTGCCC GCCCAGGAGC TCTGAGGTCT ACGCTCAGCT GTGCAACGTG GCTCGGATTG 4960
AGGCAGAGCG CGGAGCAGGG ATCCACTTCC GGCCAGGCTA TGAGTATGGC CCTGGCCTGG ACGATCTGCC TGAAAACCTC 5040
TACGGGCCCAG ATGGGGCTCC CTTCTATAAC TACCTAGGCC CCGAGGACAC TGCCCCTGAG CCTCCCTTCT CCAACCCAGC 5120
CAGCCAGCCG GGAGACAACA CACCTGTCCT TGAGCCTCCT CTGCAGCCCT CTGAACTTCA GCCTCACTAT CTAGCCAGCC 5200
ACTCAGAACC CCCTGCCTCC TTCGAAGGCC TTCAGGCTGA ATCCTGAATG GGAATGTGGC GCTGTGAGAA TGGCCGCTGC 5280
GTGCGTGTGC GGGAGGGCTA CACTTGCGAC TGCTTTGAGG GCTTCCAGCT GGATGCGCCC ACATTGGCCT GTGTGGATGT 5360
GAACGAGTGT GAAGACTTGA ACGGGCCTGC GCACCTCTGT GCACACGGTC ACTGTGAGAA CACAGAGGGT TCCTATCGCT 5440
GCCACTGTTC GCCAGGTTAC GTGGCAGAGC CAGGCCCCCC ACACTGTGCG GCCAAGGAGT AG 5502
```

FIG. 27-3

```
MESTSPRGLRCPQLCSHSGAMRAPTTARCSGCIQRVRWRGFLPLVLAVLMGTSHAQRDSIGRYEPASRDANRLWHPVGSHPAAAAKVYS          90
LFREPDAPVPGLSPSEWNQPAQGNPGWLAEAEARRPPRTQQLRVQPPVQTRRSHPRGQQQIAARAAPSVARLETPQRPAAARRGRLTGR          180
NVCGGQCCPGWTTSNSTNHCIKPVCQPPCQNRGSCSRPQVCICRSGFRGARCEEVIPEEEFDPQNARPVPRRSVERAPGPHRSSEARGSL          270
VTRIQPLVPPPSPPPSRRLSQPWPLQQHSGPSRTVRRYPATGANGQLMSNALPSGLELRDSSPQAAHVNHLSPPWGLNLTEKIKKIKVVF          360
TPTICKQTCARGRCANSCEKGDTTTLYSQGGHGHDPKSGFRIYFCQIPCLNGGRCIGRDECWCPANSTGKFCHLPVPQPDREPAGRGSRH          450
RTLLEGPLKQSTFTLPLSNQLASVNPSLVKVQIHHPPEASVQIHQVARVRGELDPVLEDNSVETRASHRPHGNLGHSPWASNSIPARAGE          540
APRPPPVLSRHYGLLGQCYLSTVNGQCANPLGSLTSQEDCCGSVGTFWGVTSCAPCPPRQEGPAFPVIENGQLECPQGYKRLNLSHCQDI          630
NECLTLGLCKDSECVNTRGSYLCTCRPGLMLDPSRSRCVSDKAVSMQQGLCYRSLGSGTCTLPLVHRITKQICCCSRVGKAWGSTCEQCP          720
LPGTEAFREICPAGHGYTYSSSDIRLSMRKAEEEELASPLREQTEQSTAPPPGQAERQPLRAATATWIEAETLPDKGDSRAVQITTSAPH          810
LPARVPGDATGRPAPSLPGQGIPESPAEEQVIPSSDVLVTHSPPDFDPCFAGASNICGPGTCVSLPNGYRCVCSPGYQLHPSQDYCTDDN          900
ECMRNPCEGRGRCVNSVGSYSCLCYPGYTLVTLGDTQECQDIDECEQPGVCSGGRCSNTEGSYHCECDRGYIMVRKGHCQDINECRHPGT          990
CPDGRCVNSPGSYTCLACEEGYVGQSGSCVDVNECLTPGICTHGRCINMEGSFRCSCEPGYEVTPDKKGCRDVDECASRASCPTGLCLNT          1080
BGSFTCSACQSGYWVNEDGTACEDLDECAFPGVCVPTGVCTNTVGSFSCCKDCDQGYRPNPLGNRCEDVDECEGPQSSCRGGECKNTEGSYQ          1170
CLCHQGFQLVNGTMCEDVNECVGEEHCAPHGECLNSLGSFFCLCAPGFASAEGGTRCQDVDECAATDPCPGGHCVNTEGSFSCLCETASF          1260
QPSPDSGECLIDECEDREDPVCGAWRCENSPGSYRCILDCQPGFYVAPNGDCIDIDECANDTVCGNHGFCDNTDGSFRCLCDQGFETSP          1350
SGWECVDVNECELMMAVCGDALCENVEGSFLCLCASDLEEYDABEGHCRPRVAGAQRIPEVRTEDQAPSLIRMECYSEHNGGPPCSQILG          1440
QNSTQABCCCTQGARWGKACAPCPSEDSVEFSQLCPSGQGYIPVEGAWTFGQTMYTDADECVLFGPALCQNGRCSNIVPGYICLCNPGYH          1530
YDASSRKCQDHNECQDLACENGECVNQEGSFHCLCNPPLTLDLSGQRCVNTTSSTEDFPDHDIHMDICWKKVTNDVCSQPLRGHHTTYTE          1620
CCCQDGEAWSQQCALCPPRSSEVYAQLCNVARIEAERGAGIHFRPGYEYGPGLDDLPENLYGPDGAPFYNYLGPEDTAPEPPFSNPASQP          1710
GDNTPVLEPPLQPSELQPHYLASHSEPPASFEGLQAEECGILNGCENGRCVRVREGYTCDCFEGFQLDAPTLACVDVNECEDLNGPARLC          1800
AHGHCENTEGSYRCHCSPGYVAEPGPPHCAAKE          1833
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FIG. 28